

CONTRACT NUMBER: N62645-12-C-4037

TITLE: Development of a FDA-Approved Pharmaceutical to Treat Noise-Induced Hearing Loss

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REPORT DATE: August 13, 2014

TYPE OF REPORT: Final

PREPARED FOR: Advanced Medical Development
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REPORT DOCUMENTATION PAGE

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1. REPORT DATE (DD-MM-YYYY) 08/13/2014			2. REPORT TYPE Final Technical Report		3. DATES COVERED (From - To) 15-Aug 2012 to 14-Aug 2014	
4. TITLE AND SUBTITLE Development of a FDA-Approved Pharmaceutical to Treat Noise-Induced Hearing Loss (NIHL)					5a. CONTRACT NUMBER N62645-12-C-4037	
					5b. GRANT NUMBER	
					5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Kopke, Richard Ewert, Donald					5d. PROJECT NUMBER	
					5e. TASK NUMBER	
					5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Hough Ear Institute 3400 NW 56th Street Oklahoma City, OK 73112					8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Advanced Medical Development Naval Medical Research Center 503 Robert Grant Avenue Silver Spring, MD 20910					10. SPONSOR/MONITOR'S ACRONYM(S) AMD NMRC	
					11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT DISTRIBUTION A: Approved for public release; distribution unlimited.						
13. SUPPLEMENTARY NOTES						
14. ABSTRACT Objective: The overall objective the work reported here was designed to obtain additional pre-clinical data required to prepare the FDA Briefing Book in support of Phase 1a and 1b studies. The technical objectives of this study have provided important efficacy and pharmacokinetic pre-clinical data in rats in support of our application for FDA clinical trials.						
15. SUBJECT TERMS Noise-induced hearing loss, pharmaceutical, pre-clinical, animal studies						
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON	
a. REPORT	b. ABSTRACT	c. THIS PAGE			AMD, Program Manager, NMRC	
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4) INTRODUCTION:

Research to date by our team has discovered that n-acetylcysteine (NAC) and HPN-07 are synergistically efficacious, when given together, in that they substantially reduce permanent hearing and auditory sensory hair cell loss in chinchilla and rats exposed to high level steady state noise, as well as in rats exposed to simulated explosion. The overall objective the work reported here was designed to obtain additional preclinical data required to prepare the FDA Briefing Book in support of Phase 1a and 1b studies. The technical objectives of this study have provided important efficacy and pharmacokinetic preclinical data in rats in support of our application for FDA clinical trials.

The relatively high levels of noise exposure used in these experiments are consistent with our previous studies which were designed to address the noise conditions on aircraft carrier decks. Typically, noise levels average 155 dB sound pressure level (SPL) during landings and takeoffs. With two levels of noise protection using ear plugs and ear muffs, the noise level would be reduced by 40 dB, resulting in a 115 dB SPL of noise exposure for personnel servicing the flight operations.

(5) BODY: Scientific and Technical Objective:**Task 1: Determine effective oral dose response.****Rationale:**

Although we have conducted multiple studies with NAC and HPN-07, the aim of these experiments was to conclusively determine the most effective dosage levels of HPN-07 and NAC using the standard rat preclinical model. Determination of an effective dose range for HPN-07 plus NAC was based on auditory brain stem response (ABR), hair cell quantification, total antioxidant capacity (TAC), and correlated plasma HPN-07 and cysteine levels.

Methods:

- Baseline ABR testing was performed and then 24 male Long-Evans pigmented rats, weighing from 360 to 420 g, were exposed to octave-band noise (OBN) centered at 14 kHz at a root-mean square level of 115 dB SPL for 1 hour in a sound proof booth to induce acute acoustic trauma (AAT).
- Drugs were administered orally 1 hour after acute acoustic trauma (AAT) and then twice daily for 2 days. To determine the boundary ranges of dosage levels, escalated dosages of HPN-07 and NAC at 150, 300 and 600 mg/kg each were administered.
- Hearing capacity was assessed at 1 hour after AAT by ABR testing and again at 21 days after AAT.
- Cochlear tissue was harvested to count auditory hair cells as endpoints at day 21 after AAT.
- We also took a sample of tail vein blood after the completion of treatment at 3 days after AAT, shortly after the last medication dose, to measure the TAC and HPN-07 and cysteine levels using high pressure liquid chromatography (HPLC). Blood cysteine levels are known to increase by about 80% after oral administration of NAC. NAC is known to be metabolized to cysteine very rapidly in the gut and liver.

Findings:

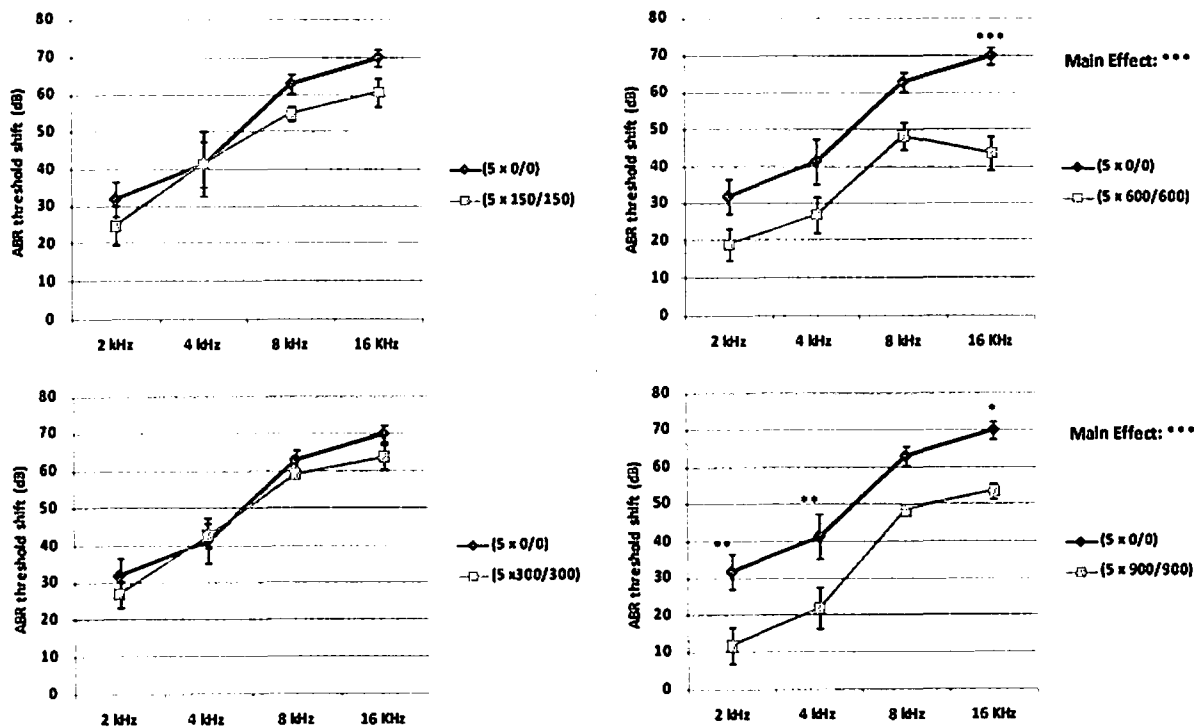
- The optimum oral drug dose for reducing hearing loss after severe noise exposure was determined to be, based on ABR testing, 5 doses of HPN-07 and NAC @ 600 mg/kg each when administered over a period of three days beginning at 1 hour after noise exposure.

- Blood Levels of HPN-07 increased proportionately with oral dose of drug. The contribution of NAC to plasma cysteine levels was masked by endogenous levels of cysteine but was detected at the two higher doses. Total antioxidant capacity of serum increased steadily with the increase in drug dose.
- The inner hair cell data for the 600/600 dosing protocol seems to be an anomaly since similar dosing in other experiments (Task 6.2 and 8.2) showed considerably more protection.

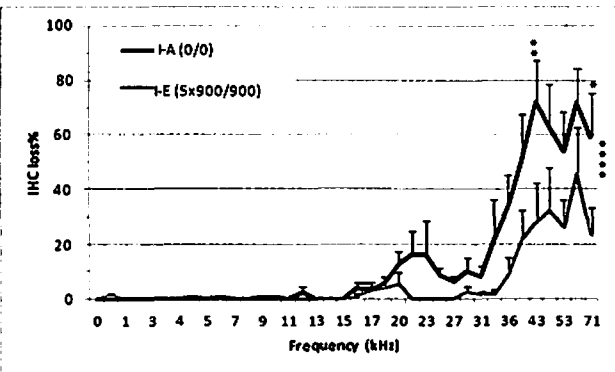
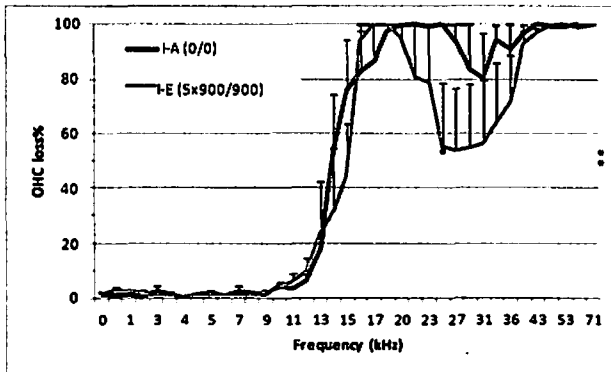
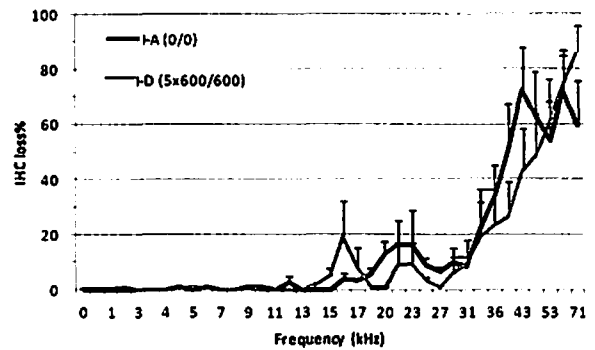
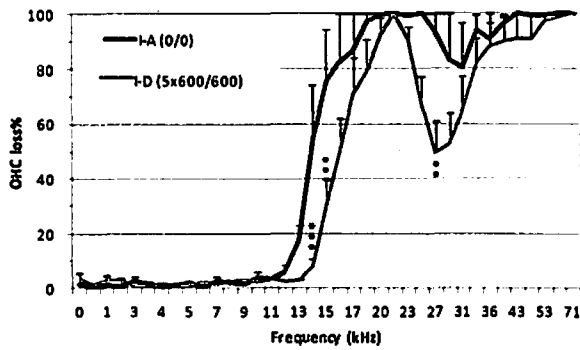
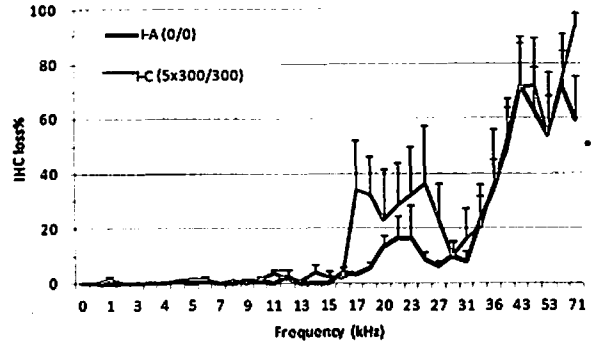
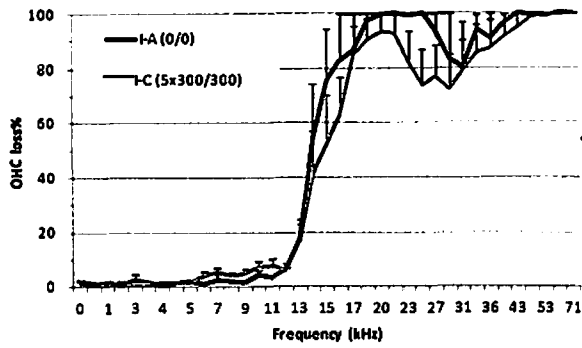
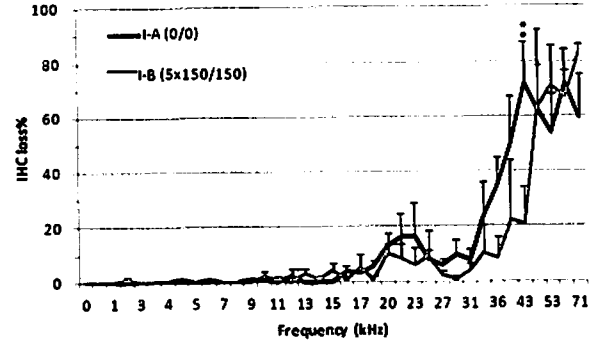
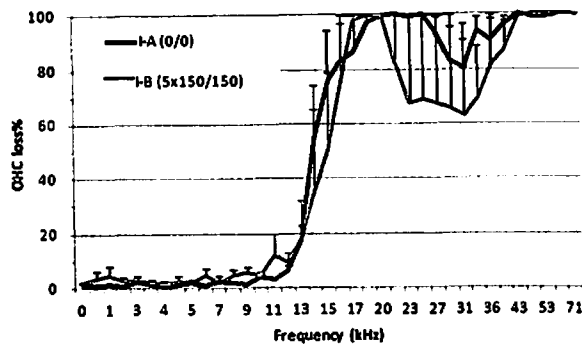
Figure Legend:

- The number of doses for 3 days: 5 (1hr, 18hr, 24hr, 42hr, 48hr).
- The dose of HPN-07/NAC in mg/kg: 0/0, 150/150, 300/300, 600/600, 900/900.
- P value: * < 0.05, ** < 0.01, *** < 0.001.

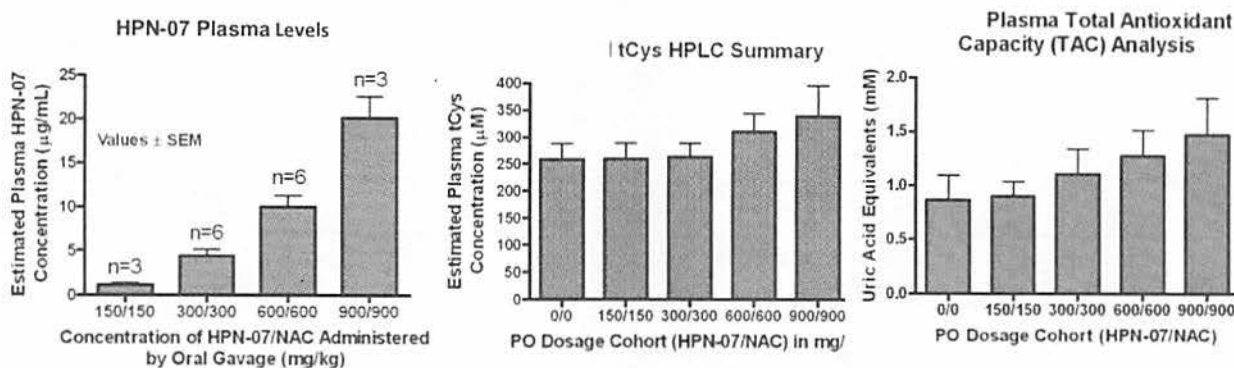
Task 1.1 ABR Results



Task 1.2 Hair cell counts



Task 1.3 Total antioxidant capacity (TAC) and HPN-07 and cysteine levels in blood 1 hour following the final dose of HPN-07/NAC. A consistent therapeutic effect was seen at the 600/600 mg/kg dose.



Task 2: Examine effect of a single dose of HPN-07/NAC.

Rationale:

A regimen of 5 doses spaced over 48 hours was established to sustain the blood levels of HPN-07 and NAC which have a relatively short half-life. However, it is important to know how much an effect one dose of drugs administered within 1 hour after AAT can have on blocking the initiation of the pathological processes that result in hearing loss. Preliminary data suggest a significant therapeutic effect even after the first dose. To this end, we administered one oral dose of HPN-07/NAC (600 mg/kg each) at one hour after AAT. The determination of the effectiveness of a single dose of HPN-07 plus NAC compared to the 5 dose regimen was determined by ABR measurement and hair cell quantification.

Methods:

- Baseline ABR testing was performed, and then 6 male Long-Evans pigmented rats, weighing from 360 to 420 g, were exposed to octave-band noise (OBN) centered at 14 kHz at a root-mean square level of 115 dB SPL for 1 hour in a sound proof booth to induce AAT.
- One oral dose of HPN-07/NAC (600 mg/kg each) was administered one hour after AAT. Hearing capacity was assessed at 1 hour after AAT by ABR testing and again at 21 days after AAT.
- In addition to ABR testing, cochlear tissue was harvested to count hair cells as endpoints at day 21 after AAT.
- These data were compared to animals receiving the full regimen of 5 doses over 3 days.

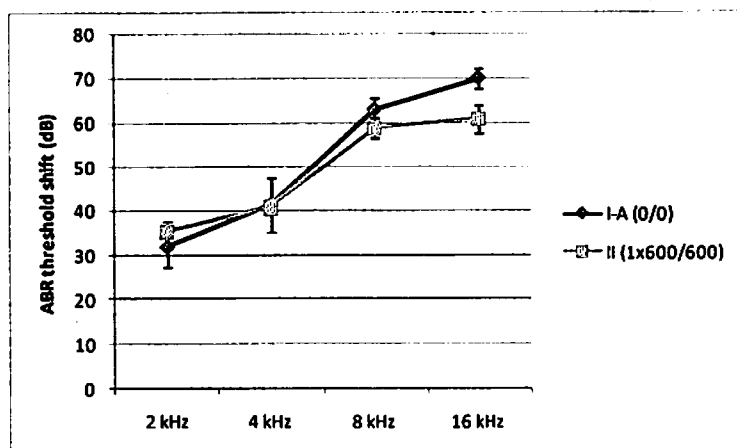
Findings:

A single dose of HPN-07 and NAC @ 600 mg/kg given 1 hour after noise exposure had no detectable effect on ABR threshold shifts but have a detectable (Main Effect) on reducing outer hair cells (OHS) and inner hair cell loss (IHS).

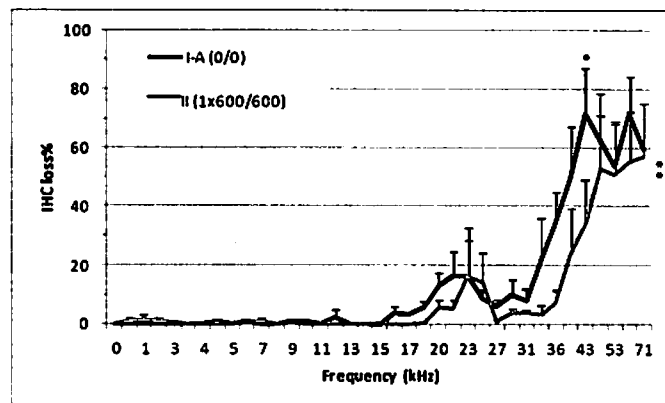
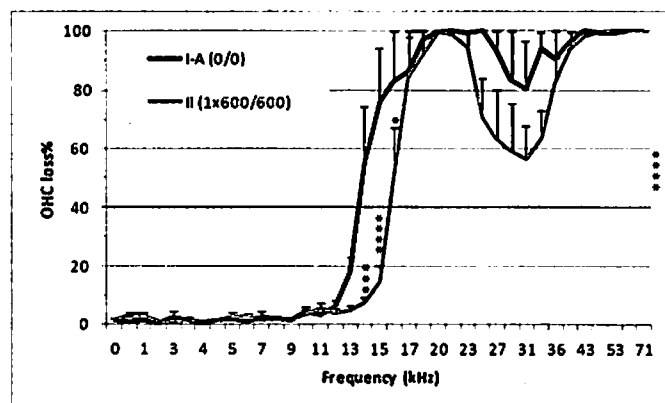
Legend:

- The number of doses: single dose at hour 1 post noise.
- The dose of HPN-07/NAC in mg/kg: 0/0, 600/600.

Task 2.1 ABR Results:



Task 2.2 Hair cell counts:



Task 3: Demonstrate the pharmacokinetics of HPN-07 and NAC after per os (PO) and intraperitoneal (IP) dosing.**Rationale:**

The questions addressed in these studies were the bioavailability (blood levels) of HPN-07 administered at four (4) increasing doses (75, 150, 300, 600 mg/kg) and via two routes of administration (oral and intraperitoneal). In addition, the effect of combining NAC with HPN-07 on bioavailability was assessed at the 300 mg/kg dose level. Total free cysteine levels were also measured following administration of 300 mg/kg NAC alone.

Methods:

- Oral dosing: 36 male Long-Evans pigmented rats, weighing from 360 to 420 g with an implanted jugular vein catheter, were given four (4) increasing doses of HPN-07 alone (75, 150, 300, 600 mg/kg), a single dose of 300 mg/kg NAC alone, or a single combinatorial dose of 300 mg/kg HPN-07 and 300 mg/kg NAC by oral gavage.
 - Blood samples were collected via a cannula inserted into the jugular vein at 30m, 60m, 90m, 120m, 4h, and 8h after drug administration.
 - Blood samples were analyzed for HPN-07 and total free cysteine (NAC groups only) levels using HPLC.
- Intraperitoneal (i.p.) dosing: 36 male Long-Evans pigmented rats, weighing from 360 to 420 g with an implanted jugular vein catheter, were given four (4) increasing doses of HPN-07 alone (75, 150, 300, 600 mg/kg), a single dose of 300 mg/kg NAC alone, or a single combinatorial dose of 300 mg/kg HPN-07 and 300 mg/kg NAC by i.p. injection.
 - Blood samples were collected via a cannula inserted into the jugular vein at 30m, 60m, 90m, 120m and 4h, and 8h after drug administration.
 - Blood samples analyzed for HPN-07 and cysteine level (NAC only group) using HPLC.

Findings: Pharmacokinetics

The time course kinetics of plasma HPN-07 concentration in blood following a single oral dose (i.e. gavage) of 75, 150, 300, or 600 mg/kg of HPN-07 alone or as a combinatorial dose of 300 mg/kg HPN-07 and 300 mg/kg NAC in Long Evans rats are summarized in Table 3.1 and Figures 3.1-3.5. Following oral administration, HPN-07 achieved mean maximum concentration levels (C_{max}) between 0.5 hour (30 minutes) and 1.5 hours post-dosing. After T_{max} , the HPN-07 plasma concentrations generally exhibited a gradual decline at mean estimated $t_{1/2}$ values ranging from 2.5 to 4.2 hours. Mean area under the curve (AUC) values for this route of administration exhibited a dose-dependent relationship with HPN-07 dose increases, albeit in a less than proportional manner at intermediate doses. Combinatorial treatment with 300 mg/kg NAC did not significantly impact the mean C_{max} value of HPN-07 in blood plasma following oral dosing at 300 mg/kg, however moderate increases in AUC values for HPN-07 were measured under these conditions.

Table 3.1 Mean Pharmacokinetic Parameters of HPN-07 in Rat Plasma Following Oral Dosing

Dose Level HPN-07 NAC	$t_{1/2}$	T_{max}^*	C_{max}	SE_ C_{max}	AUC _{0-Tlast}	SE_ AUC _{0-Tlast}	AUC _{inf}	AUC %Extrap obs
(mg/kg)	(h)	(h)	($\mu\text{g/mL}$)	($\mu\text{g/mL}$)	($\mu\text{g}\cdot\text{h/mL}$)	($\mu\text{g}\cdot\text{h/mL}$)	($\mu\text{g}\cdot\text{h/mL}$)	(%)
75 0	2.55	1	0.52	0.1	1.79	0.2	2.04	12.3
150 0	4.23	1	1.18	0.1	5.27	0.8	6.49	18.8
300 0	2.90	1	2.43	0.1	6.52	0.9	7.42	12.1
600 0	3.03	0.5	2.79	0.3	9.69	0.7	15.68	38.2
300 300	2.90	0.5	2.46	0.1	9.1	0.5	9.96	8.6

$t_{1/2}$, half-life; T_{max} , time to maximum concentration; C_{max} , maximum concentration; AUC, area under the curve;

SE, standard error of the mean

* Median time to maximum concentration

Figure 3.1: Graph of plasma HPN-07 concentration versus time (h) following oral administration of 75 mg/kg of HPN-07 (\pm SEM)

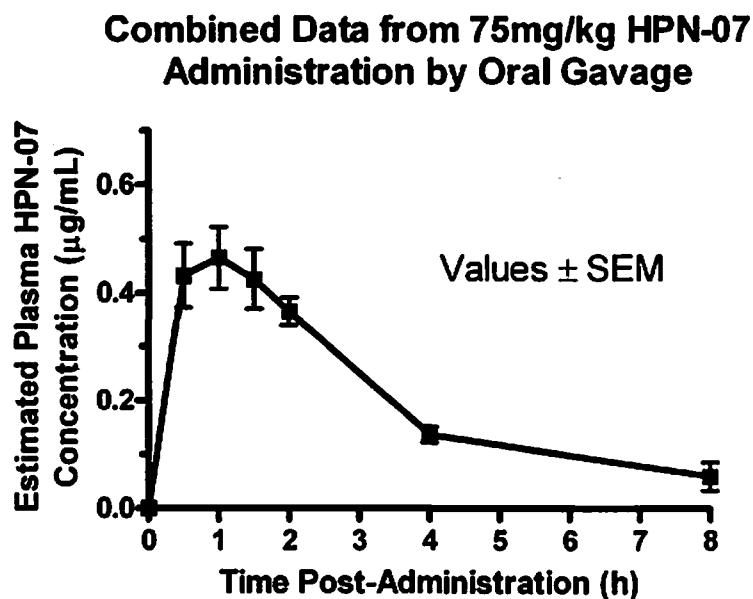


Figure 3.2: Graph of plasma HPN-07 concentration versus time (h) following oral administration of 150 mg/kg of HPN-07 (\pm SEM)

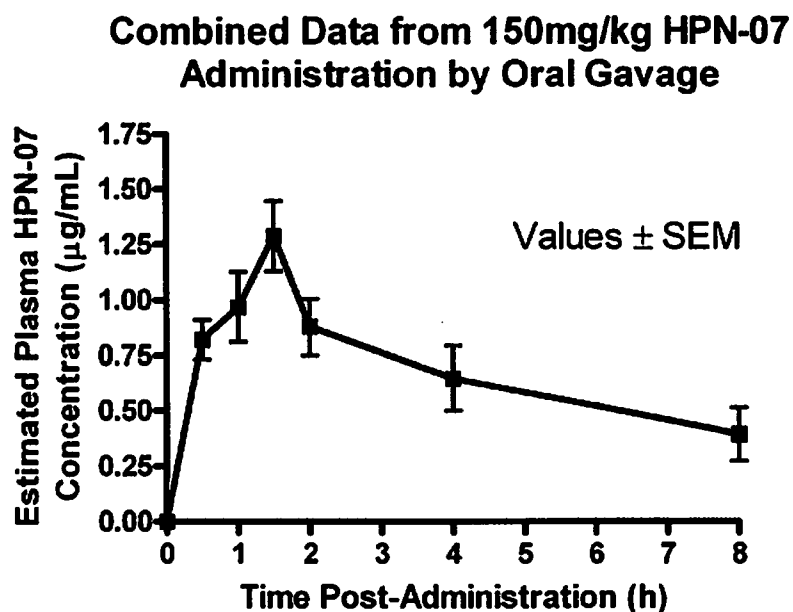


Figure 3.3: Graph of plasma HPN-07 concentration versus time (h) following oral administration of 300 mg/kg of HPN-07 (\pm SEM)

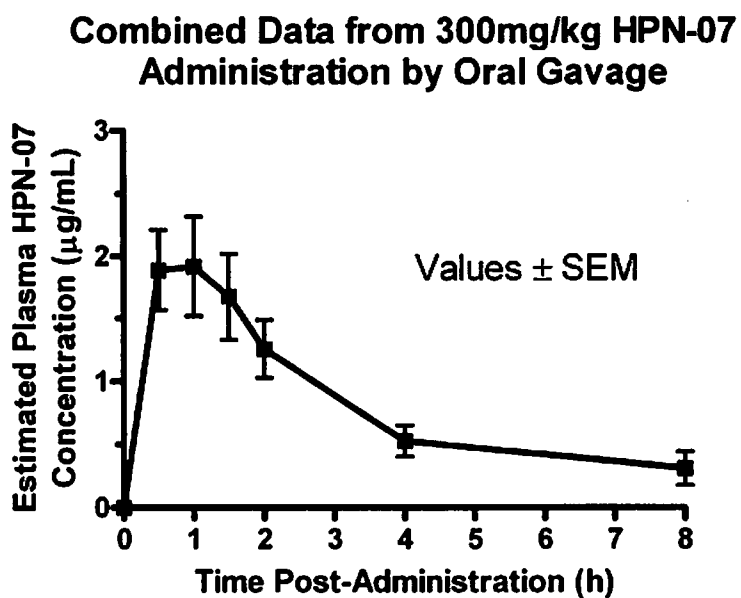


Figure 3.4: Graph of plasma HPN-07 concentration versus time (h) following oral administration of 600 mg/kg of HPN-07 (\pm SEM)

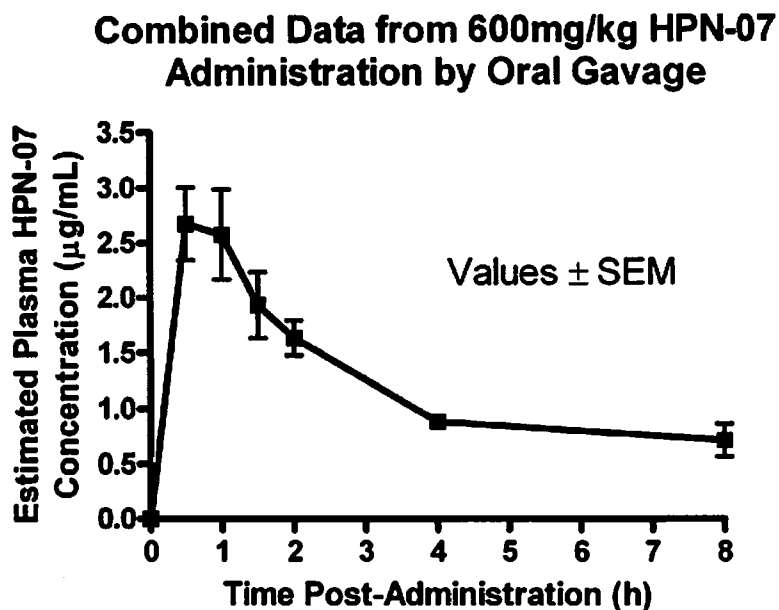


Figure 3.5: Graph of plasma HPN-07 concentration versus time (h) following oral administration of 300 mg/kg of HPN-07 and 300 mg/kg of NAC (\pm SEM)

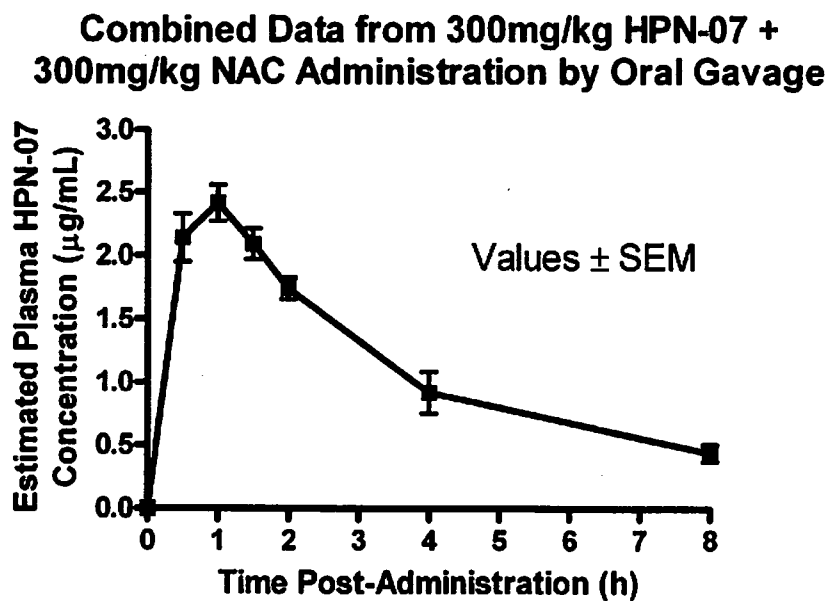


Table 3.2 Mean Pharmacokinetic Parameters of HPN-07 in Rat Plasma Following I.P. Dosing

Dose Level		$t_{1/2}$	T_{max}^*	C_{max}	$SE_{C_{max}}$	$AUC_{0-T_{last}}$	$SE_{AUC_{0-T_{last}}}$	AUC_{inf}	AUC
HPN-07	NAC	(h)	(h)	($\mu\text{g/mL}$)	($\mu\text{g/mL}$)	($\mu\text{g}\cdot\text{h/mL}$)	($\mu\text{g}\cdot\text{h/mL}$)	($\mu\text{g}\cdot\text{h/mL}$)	%Extrap obs
(mg/kg)									(%)
75	0	0.76	0.5	139.3	8.5	213.4	8.5	213.5	0
150	0	0.82	0.5	266.6	3.7	458.3	9.6	458.4	0
300	0	0.76	0.5	421.8	33.1	731.3	51.2	731.4	0
600	0	0.85	0.5	698.8	42.4	1408.5	75.6	1408.5	0
300	300	0.83	0.5	397.9	16.5	805.5	85.5	805.5	0

$t_{1/2}$, half-life; T_{max} , time to maximum concentration; C_{max} , maximum concentration; AUC, area under the curve;

SE, standard error of the mean

* Median time to maximum concentration

Figure 3.6: Graph of plasma HPN-07 concentration versus time (h) following i.p. administration of 75 mg/kg of HPN-07 (\pm SEM)

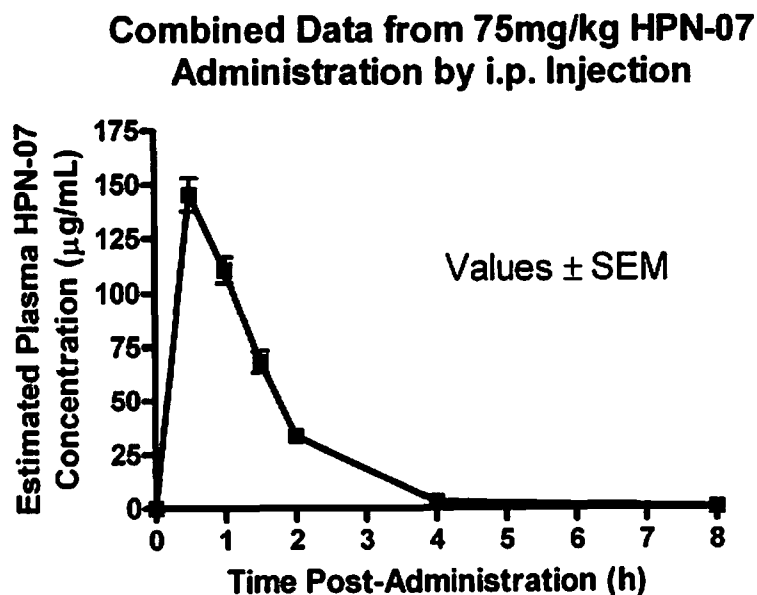


Figure 3.7: Graph of plasma HPN-07 concentration versus time (h) following i.p. administration of 150 mg/kg of HPN-07 (\pm SEM)

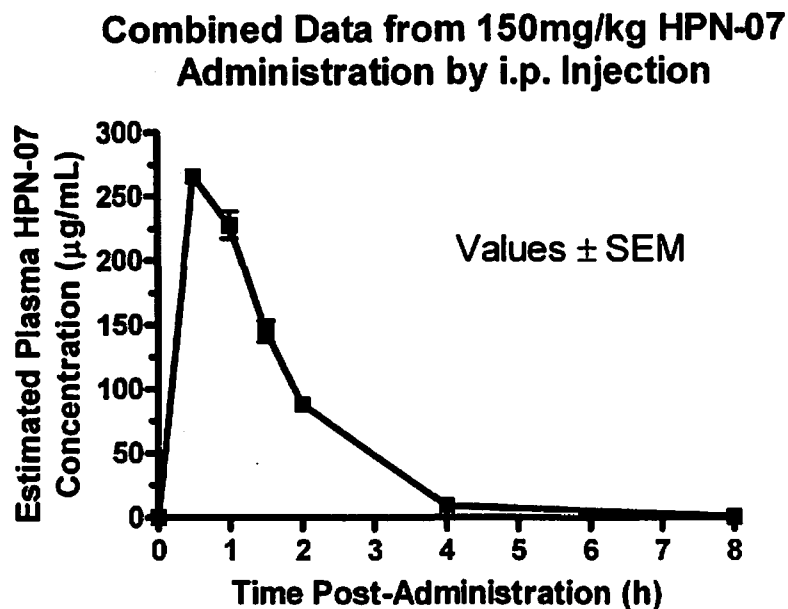


Figure 3.8: Graph of plasma HPN-07 concentration versus time (h) following i.p. administration of 300 mg/kg of HPN-07 (\pm SEM)

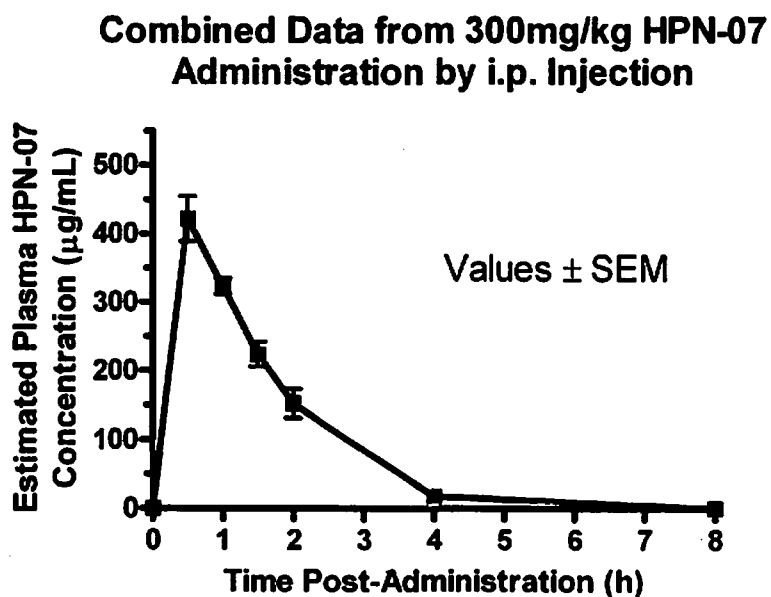


Figure 3.9: Graph of plasma HPN-07 concentration versus time (h) following i.p. administration of 600 mg/kg of HPN-07 (\pm SEM)

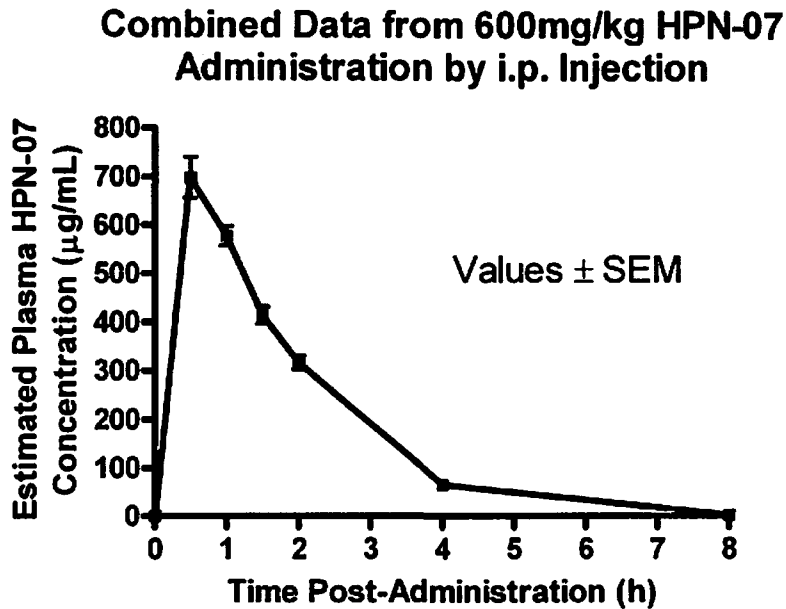


Figure 3.10: Graph of plasma HPN-07 concentration versus time (h) following i.p. administration of 300 mg/kg of HPN-07 and 300mg/kg NAC (\pm SEM)

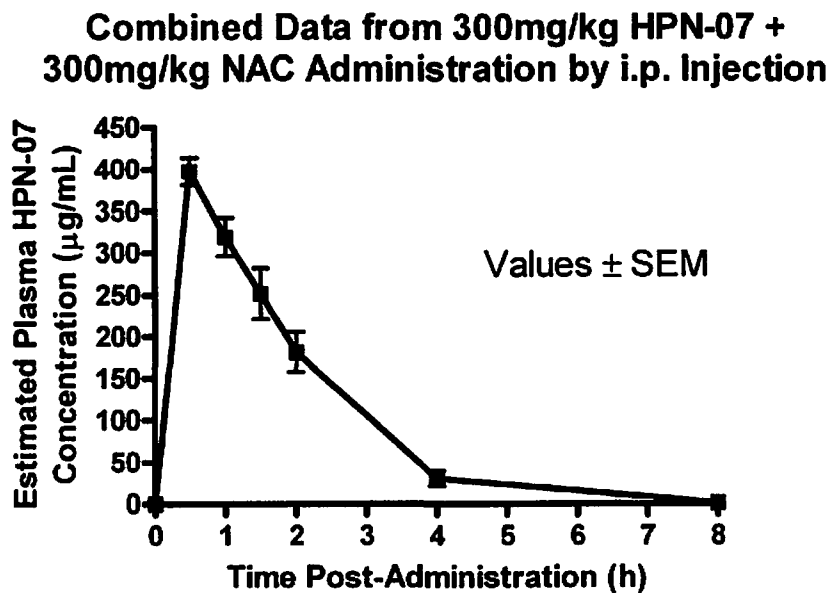


Table 3.3 Mean Pharmacokinetic Parameters t-Cys in Rat Plasma Following Oral and I.P. Dosing

Group	Dose Level		$t_{1/2}$	T_{max}^*	C_{max}	$SE_{C_{max}}$	$AUC_{0-T_{last}}^{**}$	$SE_{AUC_{0-T_{last}}}$	AUC_{inf}^{**}	$AUC\%$ %Extrap obs
	HPN- 07	NAC	(h)	(h)	($\mu\text{g/mL}$)	($\mu\text{g/mL}$)	($\mu\text{g}\cdot\text{h/mL}$)	($\mu\text{g}\cdot\text{h/mL}$)	($\mu\text{g}\cdot\text{h/mL}$)	(%)
PO	0	300	2.57	0.5	45.5	1.9	34.9	3.1	34.9	0
	300	300	2.58	1	42.1	2.3	40.0	5.4	40	0
IP	0	300	1.16	0.5	63.8	3.2	58.1	4.6	58.1	0
	300	300	1.26	0.5	60.9	2.6	49.8	4.6	49.8	0

$t_{1/2}$, half-life; T_{max} , time to maximum concentration; C_{max} , maximum concentration; AUC, area under the curve; SE, standard error of the mean

* Median time post dosing to achieve maximum concentration

** Apparent AUC calculated as measured change in tCys concentration relative to pre-dosing levels

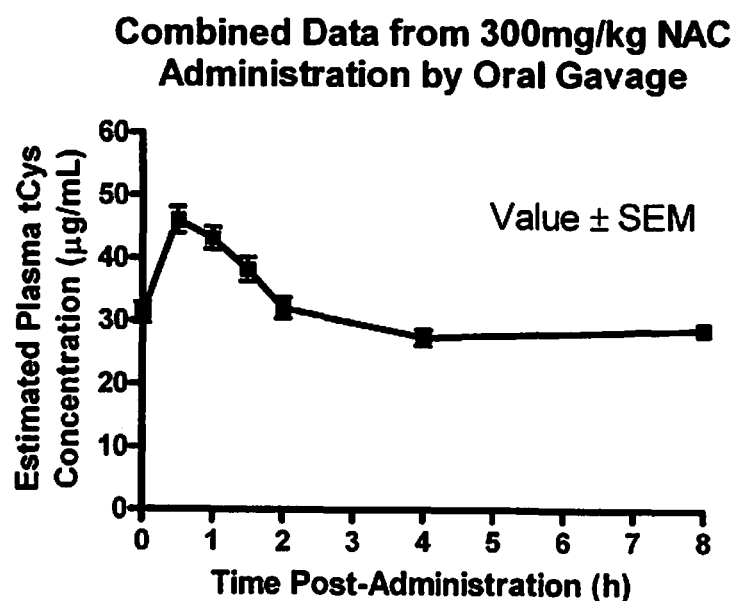
Figure 3.11: Graph of plasma tCys concentration versus time (h) following oral administration of 300 mg/kg of NAC (\pm SEM)

Figure 3.12: Graph of plasma tCys concentration versus time (h) following oral administration of 300 mg/kg of HPN-07 and 300 mg/kg NAC (\pm SEM)

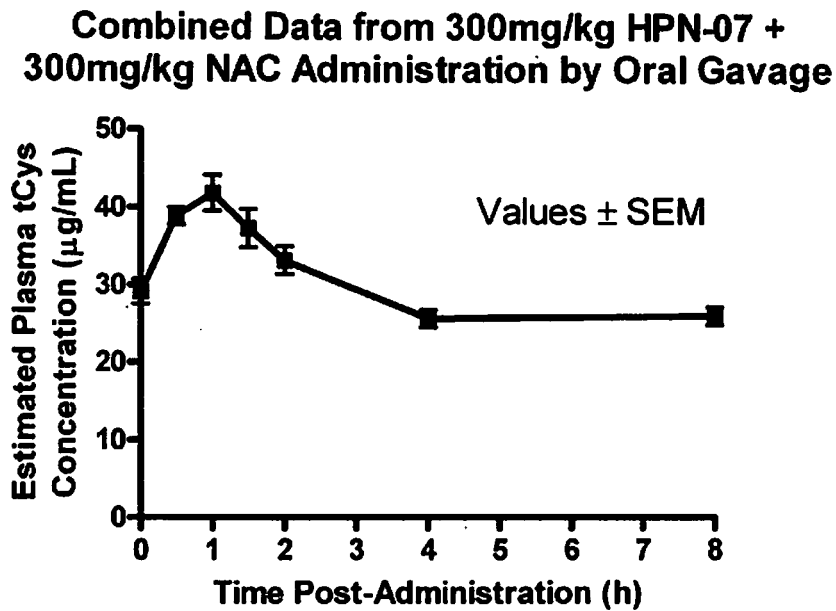


Figure 3.13: Graph of plasma tCys concentration versus time (h) following i.p. administration of 300 mg/kg of NAC (\pm SEM)

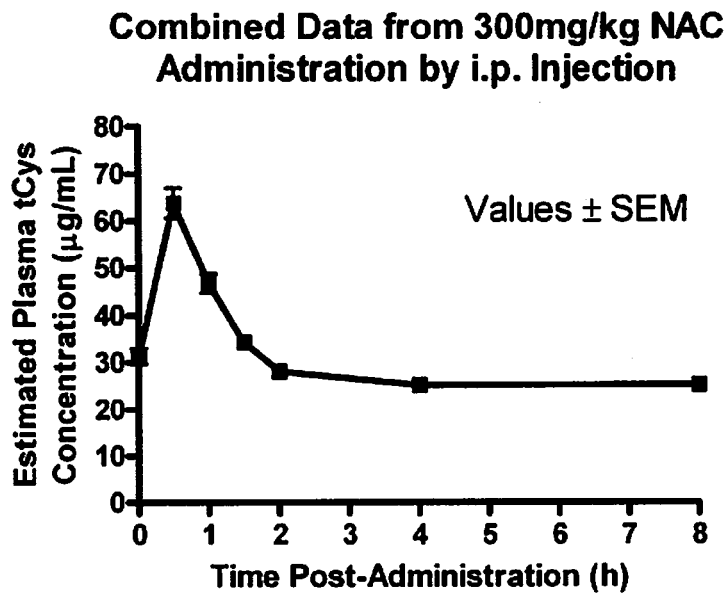
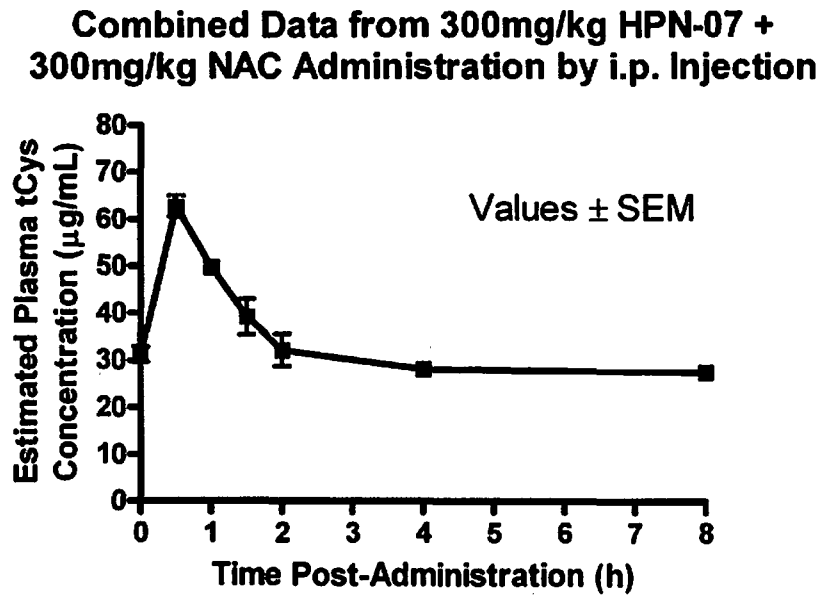


Figure 3.14: Graph of plasma tCys concentration versus time (h) following i.p. administration of 300 mg/kg of HPN-07 and 300mg/kg NAC (\pm SEM)



Task 4: Finalize the optimal oral dose ratio of HPN-07: NAC.

Rationale:

The combination of HPN-07 and NAC is known to have a synergistic effect, allowing lower effective doses of each drug. Although we have conducted multiple studies with NAC and HPN-07, it was necessary to conclusively determine the most effective dosage levels and the most effective ratios before moving into human clinical studies. The dosage of 300 mg/kg of NAC has only partial efficacy by itself. The rationale for using a ratio of HPN-07 to NAC of 1 to 2 is based on our earlier research using 4-OHPBN plus NAC where a ratio of nitron to NAC of roughly 1 to 2 was most effective. Our preliminary results indicate that HPN-07 is taken up into the blood much less than PBN after oral administration, probably because of its negatively charged sulfonyl groups. Therefore, in these experiments we tested much higher levels of HPN-07 to NAC in order to achieve higher efficacy. Our findings will allow us to determine the effect of varying the ratio of HPN-07 to NAC is altered as a function of ABR, hair cell quantification.

Methods:

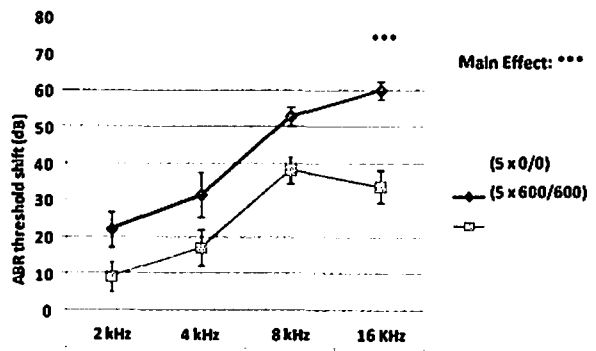
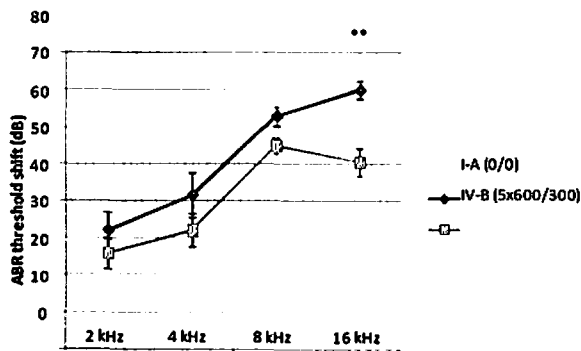
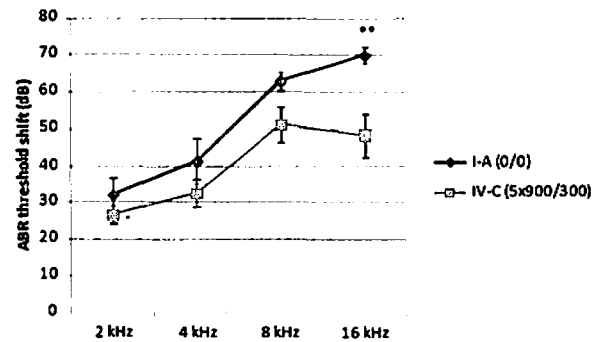
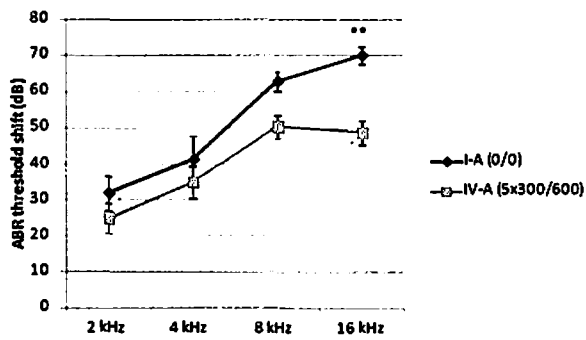
- Baseline ABR testing were performed and then 18 male Long-Evans pigmented rats, weighing from 360 to 420 g, were exposed to octave-band noise (OBN) centered at 14 kHz at a root-mean square level of 115 dB SPL for 1 hour in a sound proof booth to induce AAT.
- Doses HPN-07 and NAC in three different ratios by weight (300/600; 600/300; 900/300 mg/kg HPN-07/NAC) were administered p.o. one hour after noise exposure and twice daily for the following 2 days.
- Hearing capacity was assessed at 1 hour after AAT by ABR testing and again at 21 days after AAT.
- Cochlear tissue was taken to count hair cells as endpoints at day 21 after AAT.
- A sample of blood were collected after the completion of the HPN-07 plus NAC.

Findings:

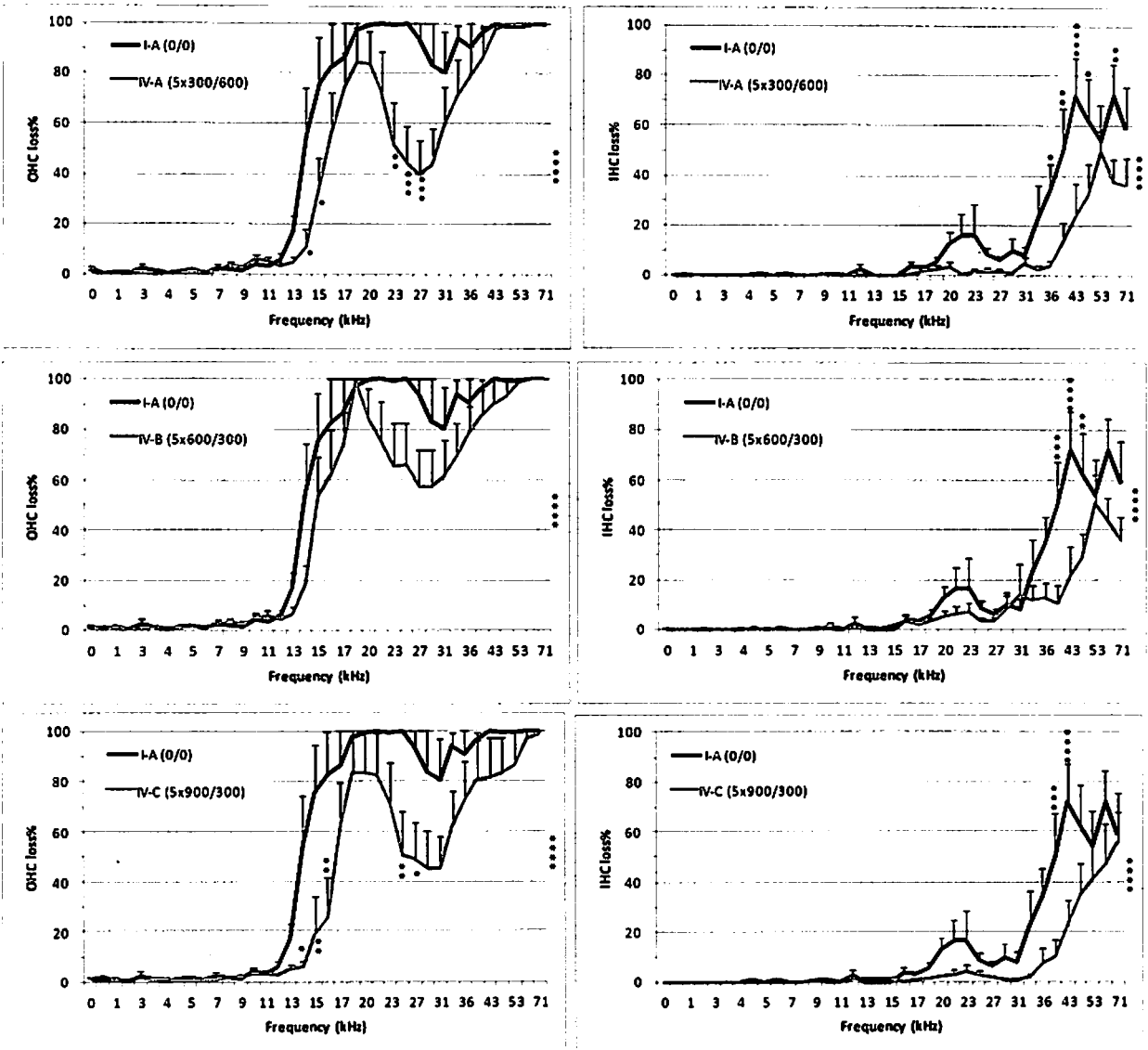
The optimum molar ratio of HPN-07/NAC for obtaining a treatment effect was found to be approximately 1:2.7.

Legend:

- The number of doses for 3 days: 5.
- The dose of HPN-07/NAC in mg/kg: (300/600; 600/300; 900/300 mg/kg HPN-07/NAC)
Figure for 600/600 dose from Task 1 was included for comparison.
- P value: * < 0.05, ** < 0.01, *** < 0.001.

Task 4.1 ABR Results.

Task 4.2 Hair cell counts



Task 5: Finalize the most effective therapeutic window for drug delivery.

Rationale:

The dosing regimens used in these experiments are those expected to be used when the therapeutic drugs (HPN-07 plus NAC) are administered in the heavy industry, occupational, or battlefield conditions. These experiments were designed to determine the effect that initial drug delivery timing, after exposure to noise, has on the amelioration of hearing loss based on ABR and cochlear hair cell count data.

Methods:

- Baseline ABR testing was performed and then 18 male Long-Evans pigmented rats, weighing from 360 to 420 g, were exposed to octave-band noise (OBN) centered at 14 kHz at a root-mean square level of 115 dB SPL for 1 hour in a sound proof booth to induce AAT.
- Drugs were administered at 600 mg/kg for both HPN-07 and NAC.
- The oral dosing protocol of 5 total doses as in previous rat experiments was used by initiating the first dosing at 4, or 12 or 24 hours after noise exposure. Hearing capacity was assessed at 1 hour after AAT by ABR testing and again at 21 days after AAT.
- Cochlear tissue was taken to count hair cells as endpoints at day 21 after AAT.
- The results were compared with the data we have obtained when the treatment regimen was initiated at 1 h post AAT.

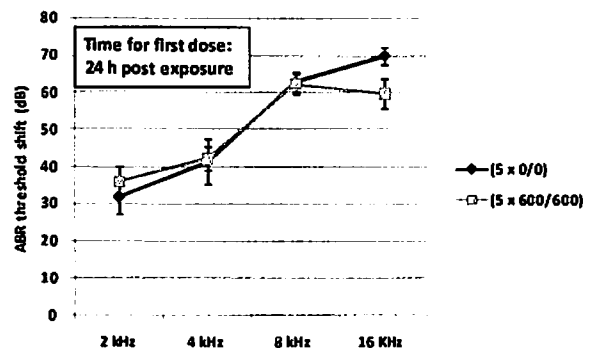
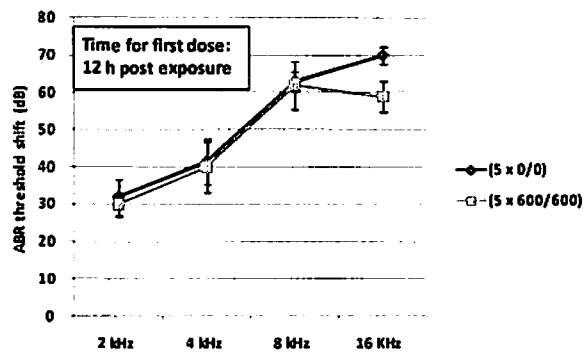
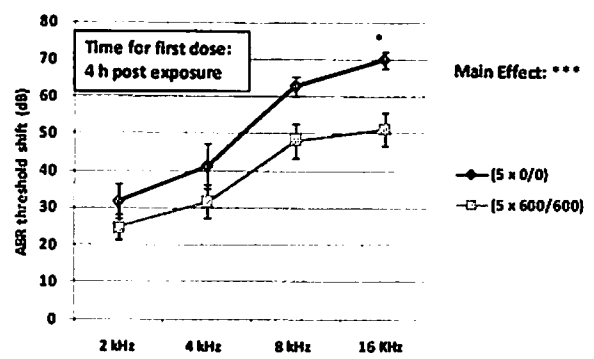
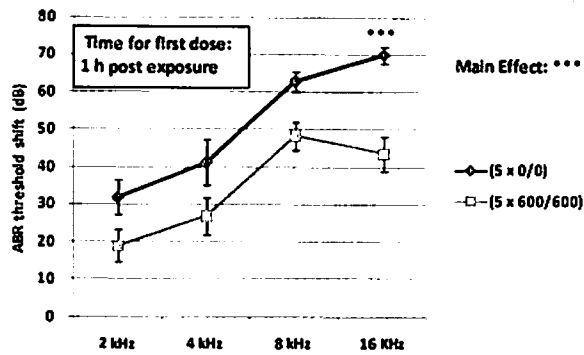
Findings:

The results indicate that the HPN-07/NAC combination did not have a detectable effect on ABR threshold shifts when administered 12 hours following noise exposure however a significant effect was observed on the reduction of inner and outer hair cell loss at every time point including the treatment that was initiated 24 hours after noise exposure.

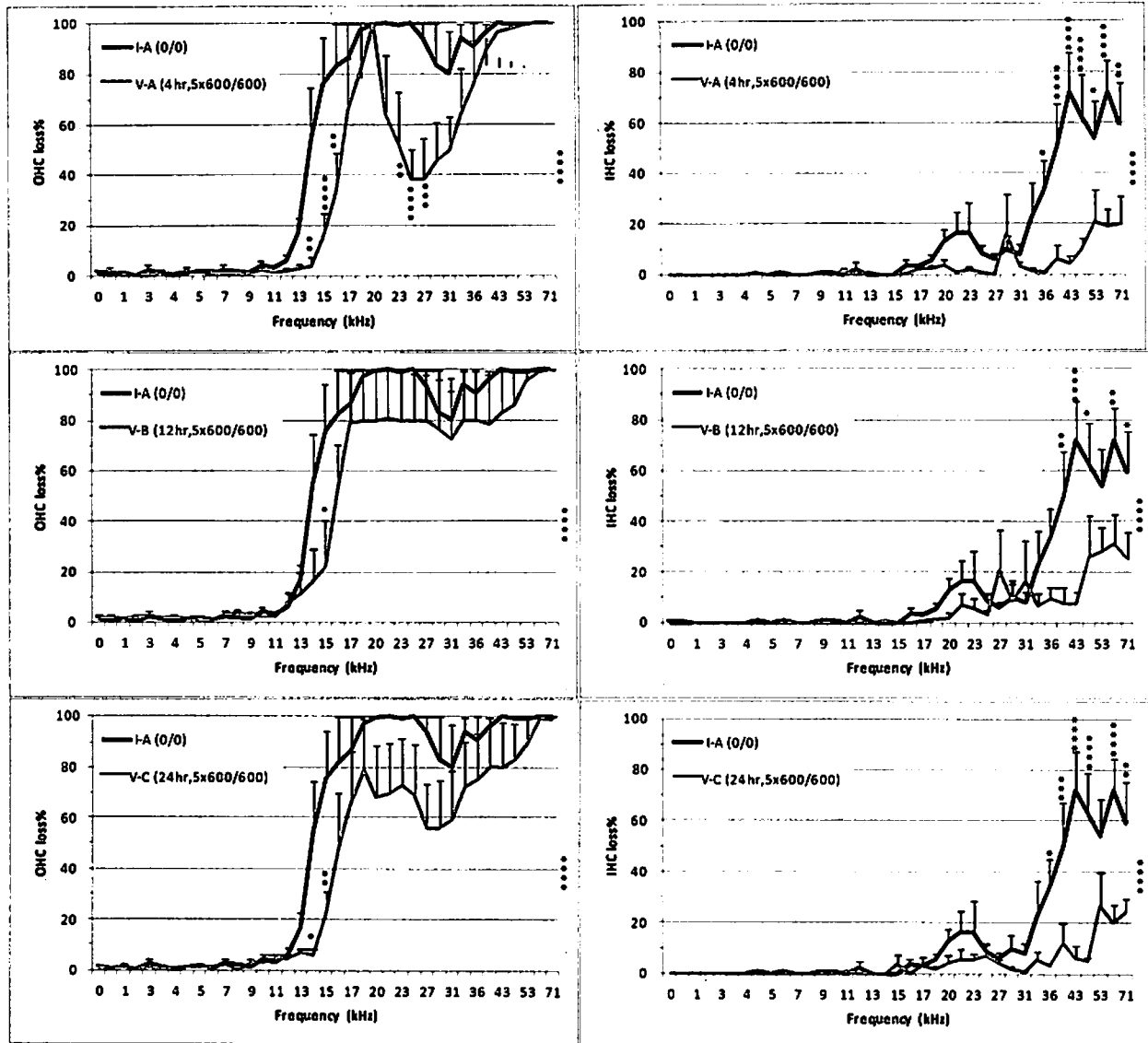
Legend:

- The time to start treatment after exposure: 1 hour, 4 hours, 12 hours, 24 hours.
- The number of doses for 3 days: 5.
- The dose of HPN-07/NAC in mg/kg: 0/0, 600/600.
- P value: * < 0.05.

Task 5.1 ABR Results



Task 5.2 Hair cell counts



Task 6: Demonstrate the pharmacodynamics of Cmax vs. AUC.

Rationale:

It is important to gain an initial understanding of the pharmacodynamics associated with the efficacy response to HPN-07 plus NAC. Therefore the aim of these experiments was to determine whether the efficacy obtained using a daily high dose of HPN-07 and NAC will perform better than lower daily doses of HPN-07 and NAC. The high dose study should yield higher Cmax (maximum concentration) values, and if the efficacy is Cmax dependent, then the high dose study should yield better efficacy than the low dose study. The extended lower dose regimen allowed us to compare the area under the curve (AUC) to Cmax results in terms of efficacy. The determination of whether AUC or Cmax is a more important determinate of treatment efficacy was based on ABR and hair cell count data.

Methods:

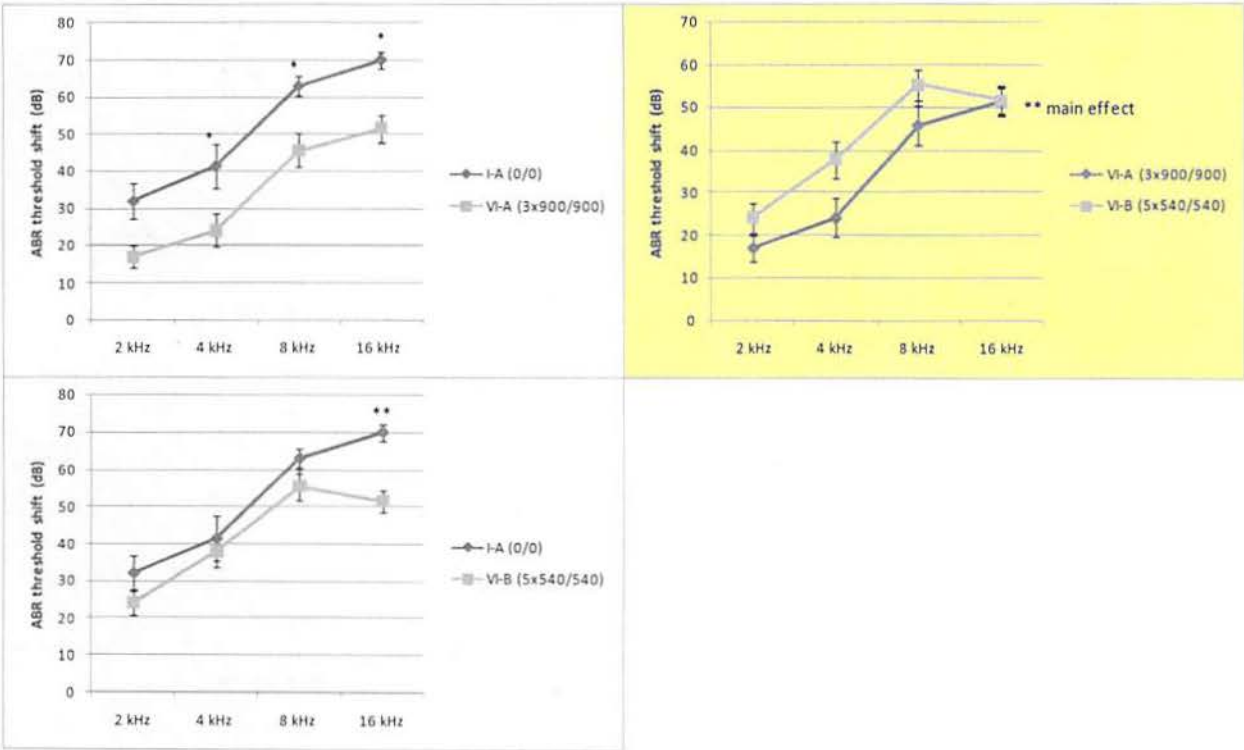
- Baseline ABR testing was performed and then 12 male Long-Evans pigmented rats, weighing from 360 to 420 g, will be exposed to octave-band noise (OBN) centered at 14 kHz at a root-mean square level of 115 dB SPL for 1 hour in a sound proof booth to induce AAT.
- After noise exposure, rats were divided into two groups: Six rats were orally administered 900 mg/kg of HPN-07/NAC at 1 hour, 18 hours, and 24 hours after noise exposure for a total dose of 2700 mg/kg; a second group of six rats were orally administered 540 mg/kg of HPN-07/NAC at 1 hour, 18 hours, 24 hours, 42 hours, and 48 hours after noise exposure for a total dose of 2700 mg/kg.
- Hearing capacity was assessed at 1.5 hours after AAT by ABR testing and again at 21 days after AAT.
- Cochlear tissues were taken to count hair cells as endpoints at day 21 after AAT.

Findings: A higher dose given over the shortest period of time had a greater effect on reduction of ABR threshold shifts and preservation of inner hair cells, suggesting that the effect is more Cmax dependent.

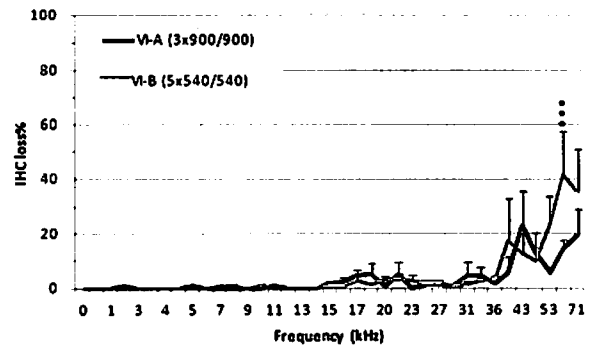
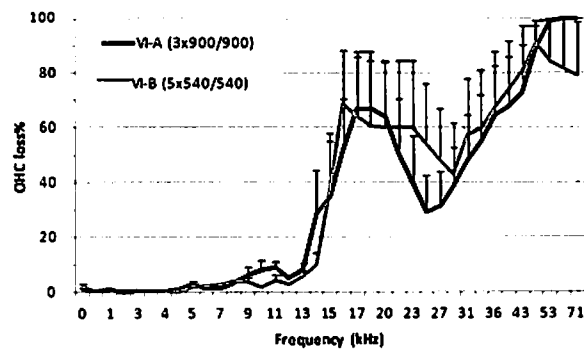
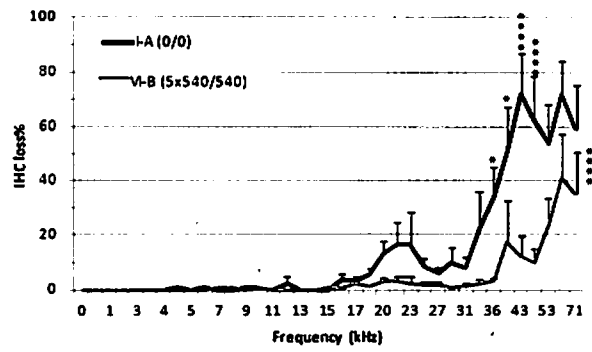
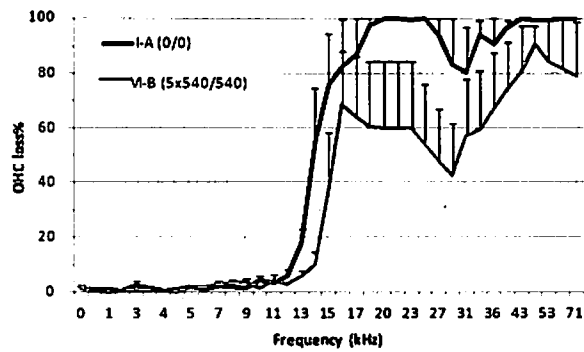
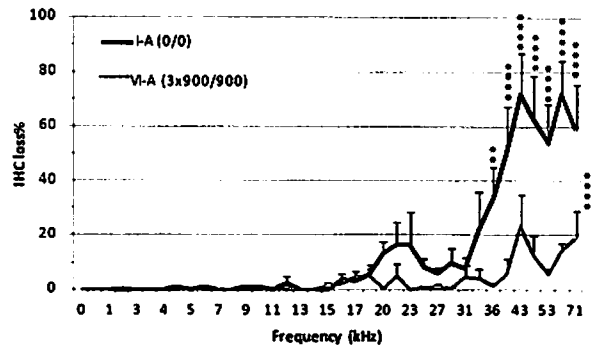
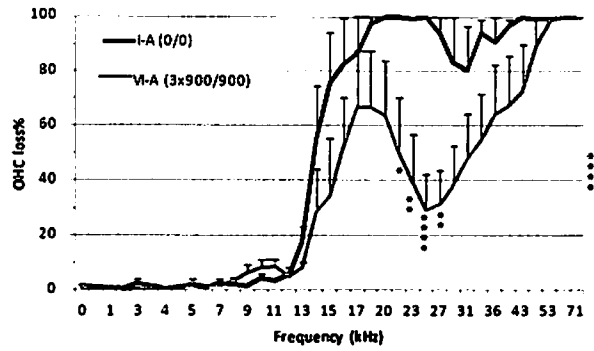
Legend:

- The number of doses* of HPN-07/NAC in mg/kg: @ 900/900: 2 days: 3 (1 hour, 18 hours, 24 hours).
- The number of doses* of HPN-07/NAC in mg/kg: @ 540/540: 3 days: 5 (1 hour, 18 hours, 24 hours, 42 hours, 48 hours).
- P value: * < 0.05, ** < 0.01, *** < 0.001.
- * total dose is same for both groups.

Task 6.1 ABR Results:



Task 6.2 Hair cell counts



Task 7: Determine the effect of a single versus combined drug treatment.

Rationale:

These studies were designed to determine the effect of oral administration of single drugs alone and compare the results to combined drug administration on reduction of hair cell and hearing loss due to AAT. They also help to reconfirm earlier findings in chinchilla exposed to steady state noise that the combination of HPN-07 plus NAC is more efficacious than either agent given alone. Reconfirmation of a synergistic effect on efficacy of the combination treatment versus treatment with each agent alone is based on ABR and hair cell count data.

Methods:

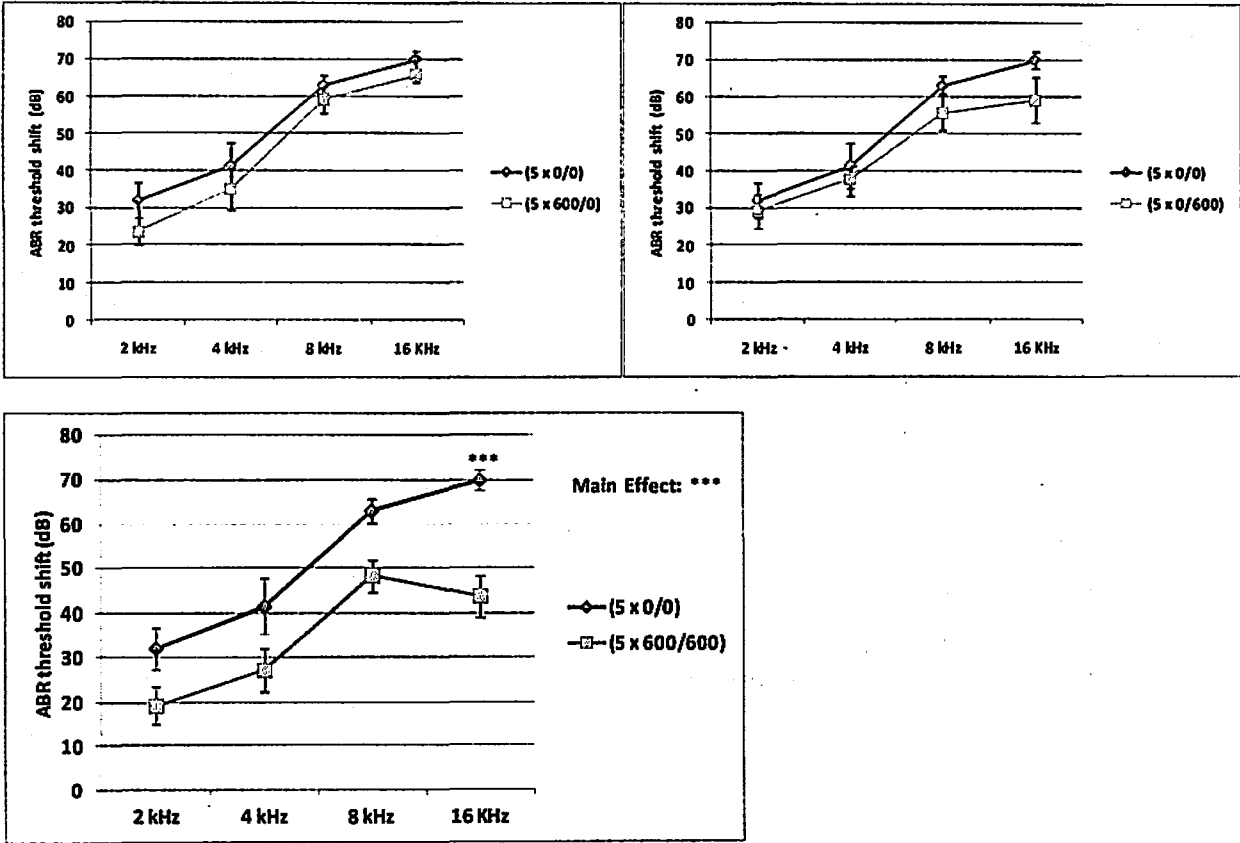
- Baseline ABR testing was performed on 24 male Long-Evans pigmented rats, weighing from 360 to 420 g.
- Noise exposure: Rats were exposed to octave-band noise (OBN) centered at 14 kHz at a root-mean square level of 115 dB SPL for 1 hour in a sound proof booth to induce AAT.
- Drugs were administered orally 1 hour after AAT and then twice daily for 2 days. The optimum dosage levels for each drug and in combination, as determined in Tasks 1 and 4, was used in these experiments.
- Hearing capacity was assessed at 1 hour after AAT by ABR testing and again at 21 days after AAT.
- Cochlear tissue was harvested to count hair cells as endpoints at day 21 after AAT.

Findings: A synergistic effect was observed: The reduction in ABR thresholds and in hair cell counts was greater for the drug combination (Task 1) than in the individual drug treatments at the same dose.

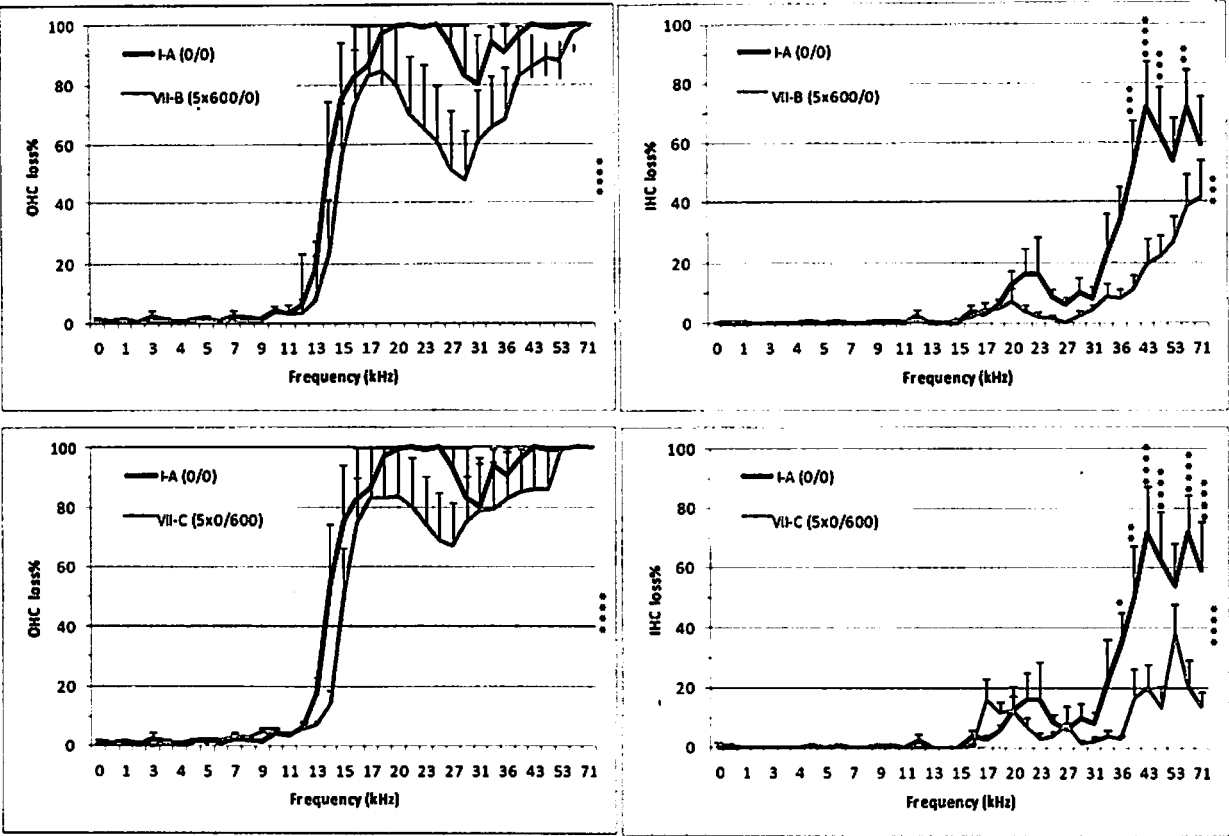
Legend:

- The number of doses for 3 days: 5.
- The dose of HPN-07/NAC in mg/kg: 0/0, 600/0, 0/600.
- P value: * < 0.05, ** <0.01, *** < 0.001.

Task 7.1 ABR Results



Task 7.2 Hair cell counts



Task 8: Effect of delivering the drugs at 4 hour intervals during the first 24 hours on reducing hearing loss.

Rationale:

The results indicate that administration of HPN-07/NAC combination after 24 hours following noise exposure did not have a detectable effect on hearing loss (Aim Five) suggesting a limited window for treatment. Based on these findings additional experiments were conducted to determine if giving 4 doses of the combination of drugs within 12 hours after noise exposure would increase the treatment effect of the drugs.

Methods:

- Baseline ABR testing was performed and then 18 male Long-Evans pigmented rats, weighing from 360 to 420 g, were exposed to octave-band noise (OBN) centered at 14 kHz at a root-mean square level of 115 dB SPL for 1 hour in a sound proof booth to induce AAT.
- We adopted the most effective dosage based on the most effective HPN-07 to NAC dose and ratio determined by the experiments put forth in Tasks 1 and 4 of 600/600 mg/kg, HPN-07/NAC.
- The oral dosing protocol of 5 total doses was initiated with the first dose at 1 hour, followed by doses at 5 hours, 8 hours, 11 hours after noise exposure.
- Hearing capacity was assessed at 1 hour after AAT by ABR testing and again at 21 days after AAT.
- Cochlear tissue was taken to count hair cells as endpoints at day 21 after AAT.
- The results were compared with the data we have obtained when the treatment regimen was initiated at 1 hour post AAT.

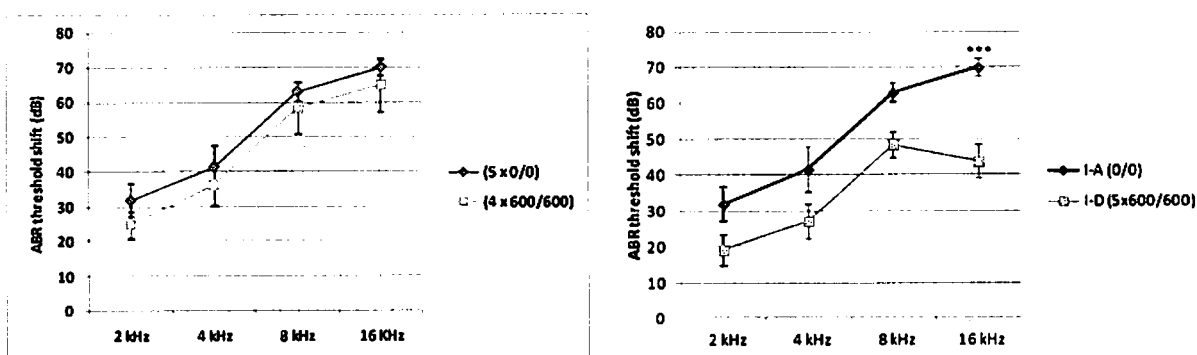
Findings:

The data indicate that concentration of drug delivery (4 doses) within a shorter period of time following noise exposure (11 hours) does not increase the therapeutic effect as measured by ABR threshold shifts. While there seems to be some positive effect for prevention of inner hair cell loss the data from the 5 dose, 3 day schedule used for comparison is questionable (see notes on Task 1).

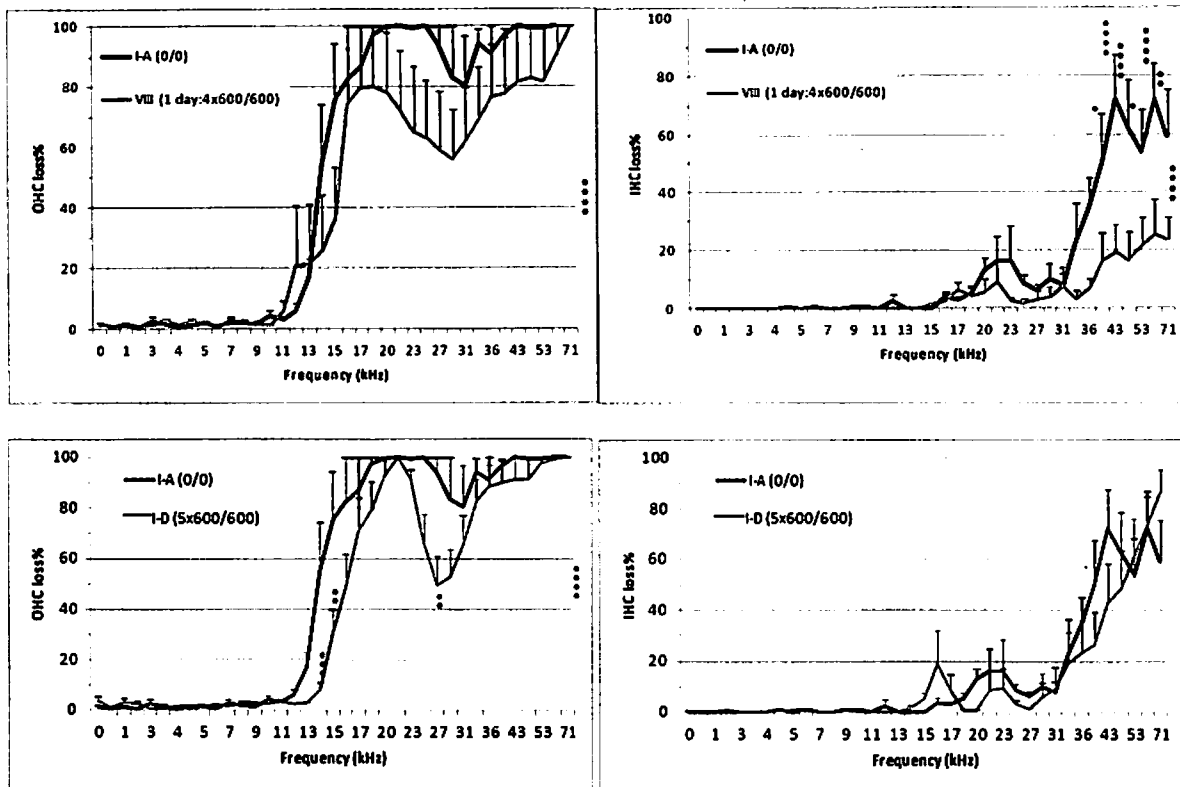
Legend:

- Dose, gavage, HPN-07/NAC (600/600 mg/kg Number of doses post noise: 4 in 11 hours (1hour, 5 hour, 8 hours, and 11 hours).
- ABR: before and about 1.5 hours (immediately after first gavage) and 21 days after noise.
- P value: * < 0.05, ** < 0.01, *** < 0.001.

Task 8.1 ABR Results: Left panes = 4 doses/11hr; Right panel= 5 doses /54hr



Task 8.2 Hair cell counts; Top panels = 4 doses/11hr; lower panels= 5 doses /54hr



Task 9. Effect of combined drug treatment using optimal drug doses and reduced noise exposure.Rationale:

One concern was that the amount of noise exposure might be extreme and thus limit our ability to detect a treatment effect of the drugs. The aim of this task was to test the effect of the drugs at a reduced duration of noise exposure that produces permanent hearing loss but that did not do as much damage to the cochlea as the intense noise levels use in the previous experiments. Exposure time was reduced from 60 to 30 minutes at the same noise levels (octave-band noise (OBN) centered at 14 kHz at a root-mean square level of 115 dB SPL).

Methods:

- Baseline ABR testing was performed and then 18 male Long-Evans pigmented rats, weighing from 360 to 420 g, were exposed to octave-band noise (OBN) centered at 14 kHz at a root-mean square level of 115 dB SPL for 1 hour in a sound proof booth to induce AAT.
- We adopted the most effective dosage based on the most effective HPN-07 to NAC dose and ratio determined by the experiments put forth in Tasks 1 and 4 of 600/600 mg/kg, HPN-07/NAC
- Hearing capacity was assessed at 1.5 hours after AAT by ABR testing and again at 21 days after AAT.
- Cochlear tissue was taken to count hair cells as endpoints at day 21 after AAT.
- The results were compared with the data obtained from rats exposed to noise for 60 minutes with the same drug dosing regimen.

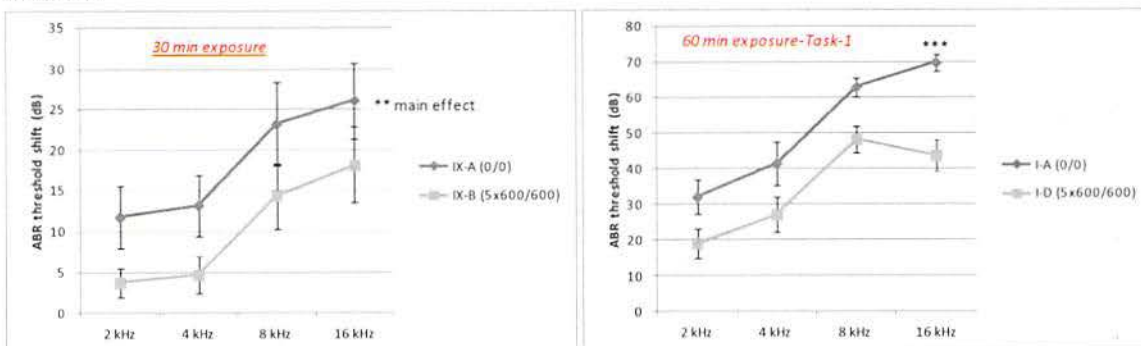
Findings:

Reducing the duration of noise exposure increased the variability of ABR readings between animals, reduced the threshold shift level at each frequency, and increased the treatment effect (except at the highest frequency). Damage to both outer and inner hair cells was reduced so that a treatment effect could not be detected.

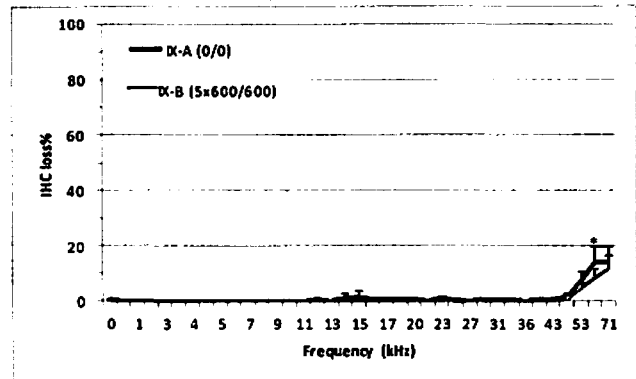
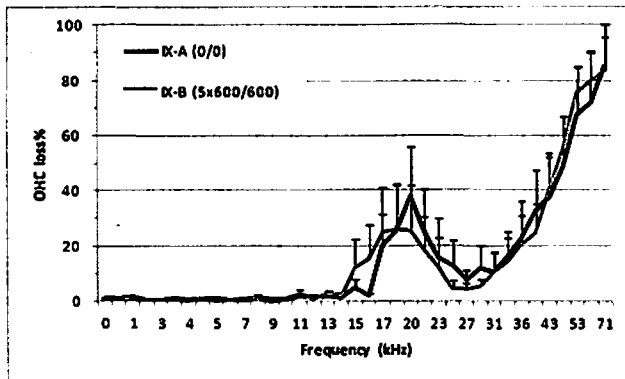
Legend:

- Noise exposure: 30 minutes- left panel; 60 minutes- right panel.
- The number of drug doses= 5 (3 days).
- The dose of HPN-07/NAC in mg/kg: 0/0, 600/600.
- P value: * < 0.05, ** < 0.01, *** < 0.001.

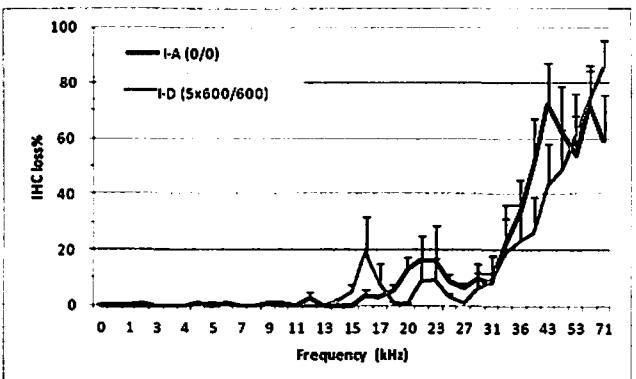
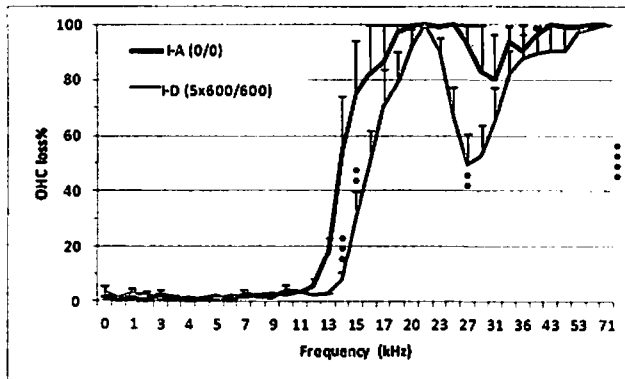
Task 9.1 ABR threshold shifts showing treatment effects following noise exposure for either 30 or 60 minutes.



Task 9.2 Hair cell counts (30 minute exposure)



Task 9.2 Hair cell counts (60 minute exposure-Task 1)



Task 10. Effects of initiating drug treatment prior to noise exposure.**Rationale:**

Considering that the timing of drug delivery relative to noise exposure is of clinical importance an experiment was designed to test the effect of giving initiating treatment 1 hour prior to noise exposure. The previous dosing schedule was maintained except that the final dose that had been given at 78 hours after noise exposure was eliminated since previous experiments demonstrated that it had little if any therapeutic effect. Therefore the total number of treatments remained the same as the previous experiments. The i.p. route of administration was used so that the data could be compared with the large amount of data collected in our previous studies and with less animal to animal variability.

Methods:

- Baseline ABR testing was performed and then 18 male Long-Evans pigmented rats, weighing from 360 to 420 g, will be exposed to octave-band noise (OBN) centered at 14 kHz at a root-mean square level of 115 dB SPL for 1 hour in a sound proof booth to induce AAT.
- Noise exposure: Octave-band noise (OBN) centered at 14 kHz at a root-mean square level of 115 dB SPL for 1 hour
- Drug dose: NPN-07/NAC: 0/0 300/300 mg/kg given by intra-peritoneal injection.
- Drug dosing schedule: 1 hour-pre-noise, and : 1 hour, 24 hours, 30 hours, and 48 hours post noise.
- Hearing capacity was assessed at 1.5 hours after AAT by ABR testing and again at 21 days after AAT.
- Cochlear tissue was taken to count hair cells as endpoints at day 21 after AAT.

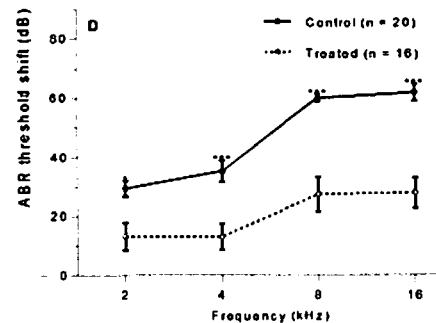
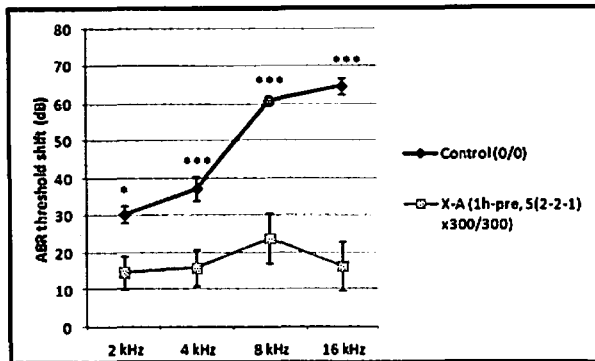
Findings:

Pre-noise treatment improved the detectable drug treatment effect only at the highest frequency (16kHz).

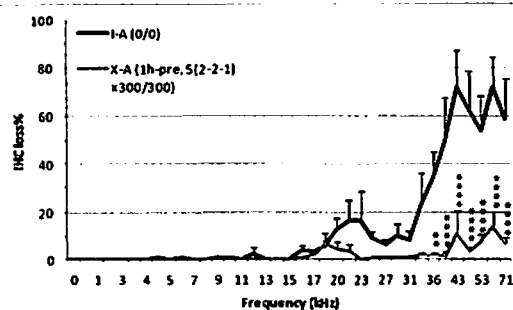
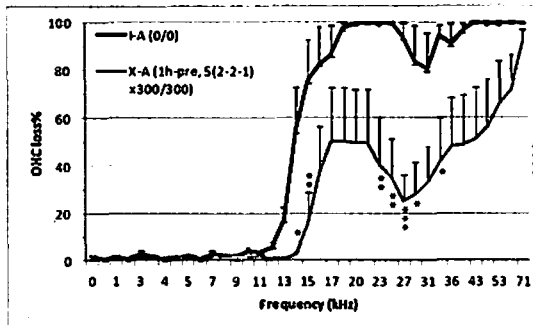
Legend:

- Drug dose: NPN-07/NAC: 0/0 300/300 mg/kg.
 - A. Dose schedule: 5 total Post noise: 1 hr, 8 hours, 24 hours, 30 hours, 48 hours (Task 11).
 - B. 1 h-pre-noise, post noise: 1 hr, 24 hours, 30 hours, 48 hours, (Task10).
- P value: * < 0.05, ** < 0.01, *** < 0.001.

Task 10.1 ABR Results: Left panel: current data; Right panel: data from previous experiments using standard 5 dose drug schedule



Task 10.2 Hair cell counts



Task 11. Effects of reducing the interval between first and second doses

Rationale:

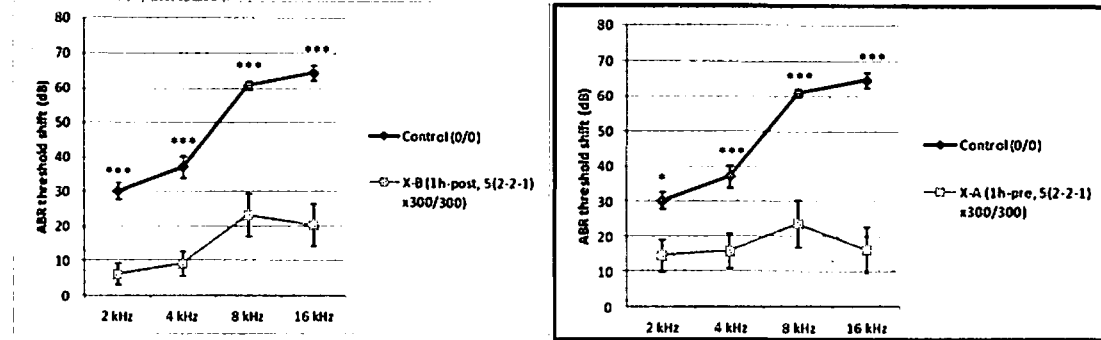
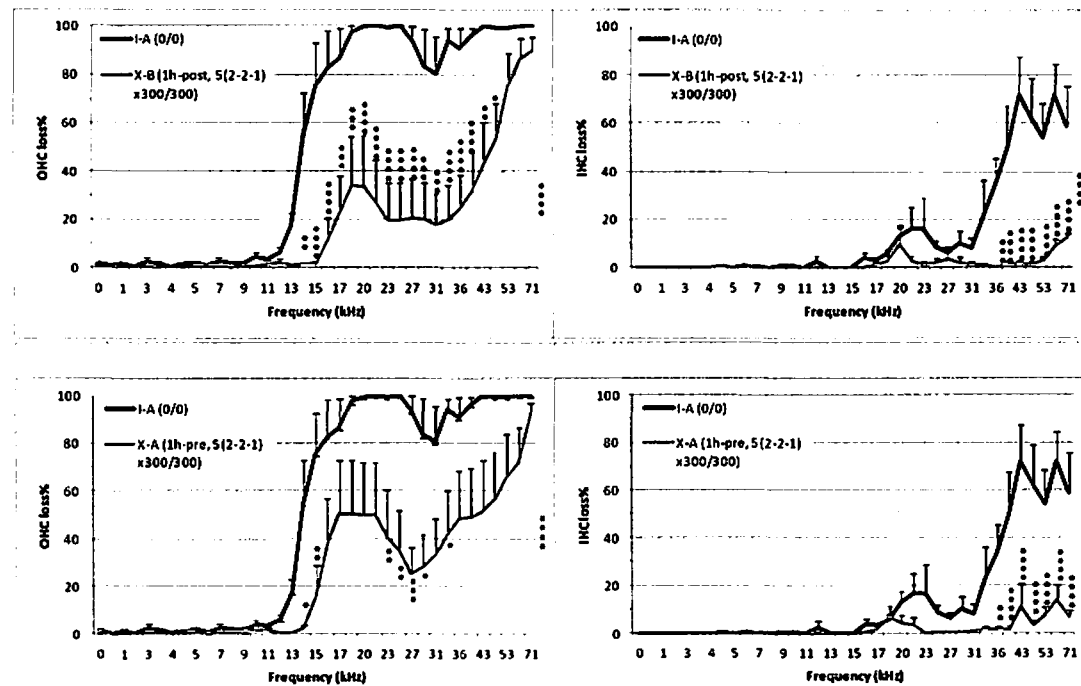
Based on our findings the maximum treatment effect required dosing within 12 hrs after noise exposure (Task 5) but that overdosing by during the same period could block the therapeutic effect (Task 8). This experiment tested the effect of reducing the interval between the first and second drug dose while keeping the remaining and total doses the same as previous experiments. Also, the total number of doses during the first 12 hours was 2 compared to the 4 in Task 8. As in Task 10, the i.p. route of administration was used so that the data could be compared with the large amount of data collected in our previous studies and with less animal to animal variability.

Methods: (same as in Task 10 except the timing of the initial two doses)

- Baseline ABR testing was performed and then 18 male Long-Evans pigmented rats, weighing from 360 to 420 g, were exposed to octave-band noise (OBN) centered at 14 kHz at a root-mean square level of 115 dB SPL for 1 hr in a sound proof booth to induce AAT.
- The intra-peritoneal dosing protocol of 5 total doses as in previously published rat experiments was used with dosing at post noise: 1 h, 8h, 24hr, 30 hr, 48 hr.
- Drug dose: NPN-07/NAC: 0/0 300/300 mg/kg
- Hearing capacity was assessed at 1h after AAT by ABR testing and again at 21 days after AAT.
- Cochlear tissue was taken to count hair cells as endpoints at day 21 after AAT.

Findings: see Figure 10.1 A and 10.2 A

The addition of a second treatment at 8h after noise exposure improved treatment effect based on ABR analysis and hair counts when compared with the effect of initiating treatment prior to noise exposure. Comparison with data from previous experiments was complicated by differences in the amount of noise exposure damage to the cochlea.

Figure 11.1 ABR threshold shift**Figure 11.2 Hair cell counts**

(6) KEY RESEARCH ACCOMPLISHMENTS:

Task 1: Determine effective oral dose response.

- Based upon ABR testing the optimum oral drug dose for reducing hearing loss after severe noise exposure was determined to be: 5 doses of HPN-07 and NAC @ 600 mg/kg each when administered over a period of three days beginning at 1 hour after noise exposure.
- Blood Levels of HPN-07 increased proportionately with oral dose of drug.
- The contribution of NAC to plasma cysteine levels was masked by endogenous levels of cysteine but was detected at the two higher doses.
- Total antioxidant capacity of serum increased steadily with the increase in drug dose.

Task 2: Examine effect of a single dose of HPN-07/NAC.

- A single dose of HPN-07 and NAC @ 600 mg/kg given 1 hour after noise exposure had no detectable effect on ABR threshold shifts but had a detectable (Main Effect) on reducing outer and inner hair cell loss.

Task 3: Demonstrate the pharmacokinetics of HPN-07 and NAC after PO and IP dosing.

- The time course kinetics of plasma HPN-07 concentration in blood following a single oral dose (i.e. gavage) of 75, 150, 300, or 600 mg/kg of HPN-07 alone or as a combinatorial dose of 300 mg/kg HPN-07 and 300 mg/kg NAC in Long Evans rats are summarized in Table 3.1 and Figures 3.1-3.5. Following oral administration, HPN-07 achieved mean maximum concentration levels (C_{max}) between 0.5 hour (30 minutes) and 1.5 hours post-dosing. After T_{max} , the HPN-07 plasma concentrations generally exhibited a gradual decline at mean estimated $t_{1/2}$ values ranging from 2.5 to 4.2 hours. Mean area under the curve (AUC) values for this route of administration exhibited a dose-dependent relationship with HPN-07 dose increases, albeit in a less than proportional manner at intermediate doses. Combinatorial treatment with 300 mg/kg NAC did not significantly impact the mean C_{max} value of HPN-07 in blood plasma following oral dosing at 300 mg/kg, however moderate increases in AUC values for HPN-07 were measured under these conditions.

Task 4: Finalize the optimal oral dose ratio of HPN-07: NAC.

- The optimum molar ratio of HPN-07/NAC for obtaining a treatment effect was found to be approximately 1:2.7.

Task 5: Finalize the most effective therapeutic window for drug delivery.

- The results indicate that the HPN-07/NAC combination did not have a detectable effect on ABR threshold shifts when administered 12 hours following noise exposure; however, a significant effect was observed on the reduction of inner hair cell loss at every time point including the treatment that was initiated 24 hours after noise exposure. Therefore these data suggest that the therapeutic window is 24 hours after noise exposure.

Task 6: Demonstrate the pharmacodynamics of Cmax vs. AUC.

- A higher dose given over the shortest period of time had a greater effect on reduction of ABR threshold shifts and preservation of inner hair cells, suggesting that the effect is more Cmax dependent.

Task 7: Determine the effect of a single versus combined drug treatment.

- A synergistic effect was observed: The reduction in ABR thresholds and in hair cell counts was greater for the drug combination (Task 1) than in the individual drug treatments at the same dose.

Task 8. Effect of delivering the drugs at 4hr intervals during the first 24hrs on reducing hearing loss.

- The data indicate that concentration of drug delivery (4 doses) within a shorter period of time following noise exposure (11 hours) does not increase the therapeutic effect as measured by ABR threshold shifts. While there seems to be some positive effect for prevention of inner hair cell loss the data from the 5 dose, 3 day schedule used for comparison is questionable (see notes on Task 1).

Task 9. Effect of combined drug treatment using optimal drug doses and reduced noise exposure.

- Reducing the duration of noise exposure increased the variability of ABR readings between animals, reduced the threshold shift level at each frequency, and increased the treatment effect (except at the highest frequency).

Task 10. Effects of drug treatment prior to noise exposure.

- Pre-noise treatment improved the detectable drug treatment effect only at the highest frequency (16kHz).

Task 11. Effects of reducing the interval between first and second doses.

- There was a detectable improved treatment effect on ABRs at all frequencies for the group receiving an additional dose of drugs at 8h after the initial dose on the day of noise exposure. The hair cell count data indicates that addition of a drug dose at 8 hours post noise had a greater treatment effect than pre-noise dosing.

(7) REPORTABLE OUTCOMES:

- The primary purpose of this research was to obtain preclinical data required to prepare the FDA Briefing Book in support of a Phase 1b study.

(8) CONCLUSION:

- These studies provided data to help establish the following: effective oral dose response; effect of a single dose of HPN-07/NAC; pharmacokinetics of HPN-07 and NAC after oral and intra-peritoneal dosing; optimal oral dose ratio of HPN-07 to NAC; most effective therapeutic window, frequency and timing for drug delivery. Our results show that: 1) dosing should be the highest safe dose associated with efficacy administered ASAP after noise exposure; 2) therapeutic window studies indicate the earlier the intervention the better the efficacy; however, even when administered 4 hours after acute high level noise exposure, efficacy was still evident; 3) optimal mg/kg dosing ratio is approximately 1:1 HPN-07:NAC, but efficacy was still evident at 1:2, 2:1, and 3:1 ratios, suggesting tolerance to variations away from 1:1; 4) pharmacokinetic studies indicate achieving high plasma drug levels (Cmax) is more important than maintaining long-term plasma drug levels (AUC). This data will be used to support a Phase 1b multiple ascending dose study in humans.

(9) REFERENCES:

None

(10) APPENDICES:

None

(11) SUPPORTING DATA:

None: all figures and tables are included in Section 5.